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Some Mycological Aspects of Penicillin Production
D. PERLMAN 449

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THE BOTANICAL REVIEW

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SOME MYCOLOGICAL ASPECTS OF PENICILLIN PRODUCTION [1]

D. PERLMAN [2]

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INTRODUCTION

While numerous reports and reviews have appeared during the past few years discussing various methods of administration and clinical application of the various penicillins, and while the recent summaries concerning the chemistry of the penicillins (37, 41) provide an index to the voluminous literature on the subject, the comparable summaries available of the studies on the mycological

[1] This review was written during December, 1949, and an effort has been made to include most of the literature appearing prior to that date. Several months later the study by Florey et al. (73a) became available and should be consulted for a more extensive treatment of some of the topics.

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production of penicillin do not include many of the aspects of that production. During the past war distribution of information concerning penicillin chemistry and manufacture was restricted by government directive, and often the reports appearing in the literature during that period, while of general interest, had little practical application. Some of this hitherto restricted information has been reviewed by Foster (88*a*) and Raper (196, 198), together with those authors' personal experiences, and other reports concerning microbiological aspects of the production of penicillin and other antibiotics are now available. The following review will draw on some of these and other sources of information to illustrate the changes in techniques which occurred during the development of the fermentation phase of the penicillin industry during the past ten years. It is unfortunate that no coordinated effort has been made by the group of laboratories which worked cooperatively during the period of 1942–45 to publish a summary of their findings, as has been done for the chemistry of the penicillins, for even the negative results would be of interest to students of microbiology.

Perhaps this has been due in part to the voluntary restrictions covering secrecy of industrial developments and practices which necessarily operate in the highly competitive chemical and pharmaceutical industries. In order to overcome this deficit, reference will be made to some of the patent literature which reflects in part some of the industrial practices. It is recognized that while this source of information is often relatively ancient history—five years have at times elapsed between application for patents and the granting of them—and frequently misleading, it has some value in indicating which practices have been tested and perhaps used in industry (105).

Recovery of the penicillin formed during the fermentation phase will not be discussed in this review, and readers are directed to the publications of Whitmore et al. (253) and others (41, 54, 32, 97) for information on this subject. This operation is fully as complicated as the fermentation phase, and, as may be seen from a flow sheet of a process formerly used by Gordon et al. (97), many steps are involved in recovering the small amounts of antibiotic from the fermented medium. While this process (Fig. 1) is perhaps more complex than those now used, it indicates that a small

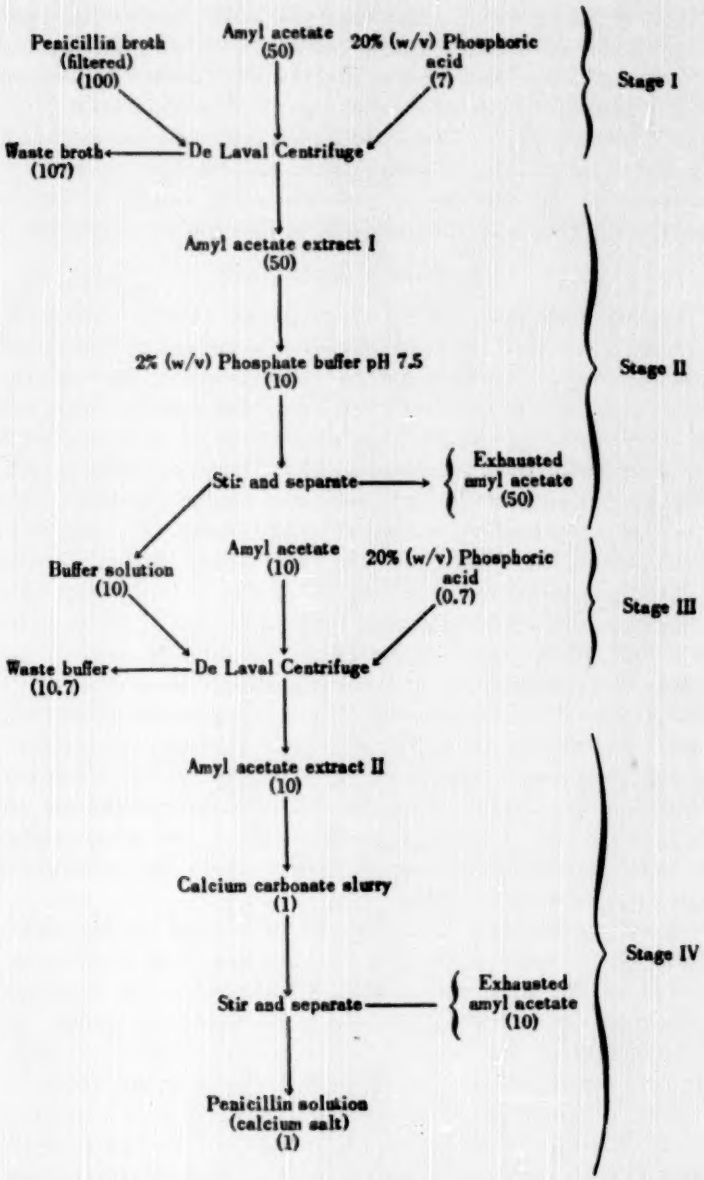


FIG. 1. Recovery of penicillin from fermented media. The numbers in parentheses refer to volumes of solution in liters. (Reprinted through courtesy of Drs. T. White and J. J. Gordon (97)).

change in the type of medium employed in the fermentation phase might upset the whole recovery process by causing emulsions in the solvent extractions or changing the distribution of the antibiotic between the solvents and water or buffer phases. This aspect of the production of penicillin has often been overlooked by those studying the fermentation phase, and perhaps explains why problems in the recovery of penicillin must always be kept in mind when new media or fermentation changes are suggested.

HISTORICAL BACKGROUND

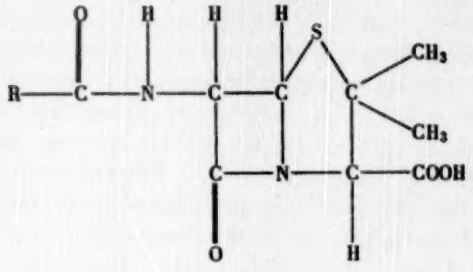
Slightly more than 20 years have passed since Dr. Alexander Fleming (70) observed that colonies of a culture of *Penicillium*, first identified as *P. rubrum* but later classified as *P. notatum* (43), growing on an agar plate inhibited the growth of adjacent colonies of *Staphylococcus*. While this observation is of considerable historical importance, the founding of the large penicillin industry perhaps dates more directly from the studies of the Oxford University group which, as related by Dr. Florey (72), started its investigations in 1938. In the intervening period between Fleming's studies and the first report by the Oxford group (36), publications by Clutterbuck et al. (43) and Reid (202) indicated that study of the active bacteriostatic agents elaborated by this culture of *P. notatum* would present problems, since those agents were so unstable chemically and did not represent major metabolic products of the mold. Indeed these investigators were uncertain whether they were studying one or many factors, and it was considered possible that a protein or similar complex substance was under study (202). For a fuller description of the events leading up to and including the Oxford studies consult the authoritative report by Florey et al. (73*a*).

Fleming applied the name "penicillin" to the culture filtrate containing the active agent (70, 71), but this name was later restricted to the antibiotic substances present in the fermented medium which were produced during growth of the fungus. As will be discussed later in this review, several structurally related chemical entities are formed by most of the penicillin-producing cultures. These are often termed the "natural penicillins" and are usually called by trivial names, e.g., penicillin-G and penicillin-X, rather than by their chemical names, viz., benzyl penicillinate and

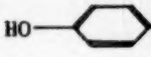
p-hydroxy benzyl penicillinate. The relationships of these "natural penicillins" to each other are apparent from their formulae presented in Table I. Since many of the early studies did not indicate which of the penicillins was under investigation, the term "penicillin" will be used here to indicate the antibiotic activity as

TABLE I

FORMULAE OF SOME OF THE NATURALLY OCCURRING PENICILLINS



β-lactam structure

R	trivial names	antibiotic activity against *Staphylococcus aureus*
		units per milligram
$CH_3-CH_2-CH=CH-CH_2-$	Penicillin I; Penicillin F.	1500
$CH_3-CH_2-CH_2-CH_2-CH_2-$	Gigantic acid; Dihydro penicillin F.	1500
	Penicillin II; Penicillin G.	1667
	Penicillin III; Penicillin X.	950
$CH_3-(CH_2)_5-CH_2-$	Penicillin IV; Penicillin K.	2250

measured by inhibition of growth in *Staphylococcus aureus*. In fact it was not until after crystallization of benzyl penicillinate (penicillin-G) had been achieved in 1943 (41, 255) that definite evidence was obtained that more than one type of penicillin is produced by the mold. Many of the fungus cultures studied were found to produce an enzyme which released hydrogen peroxide and

whose antibiotic activity was often confused with that of penicillin. Discussion of the properties of this enzyme is outside the scope of this review, and the reader is referred to the literature for a description of this enzyme, often called "notatin", "penicillin-B" and "penatin" (52, 53, 131–133, 204), but which seems identical with the glucose aerodehydrogenase studied by Müller in 1928 (166, 167). The presence of this enzyme in filtrates from fermentations does not affect the determination of penicillin content by the agar diffusion assay method (described below) and has not complicated interpretation of data collected by this method.

The success of the preliminary studies in treatment of bacterial infections reported by the Oxford group (36, 2) indicated the need for expansion of penicillin production facilities and the study of penicillin as a potential means of treating war-incurred injuries. This need was first recognized in Great Britain and resulted in the formation of the Therapeutic Research Corporation whose membership included representatives of the Boots Pure Drug Company, Ltd., British Drug Houses, Ltd., Glaxo Laboratories, Ltd., May and Baker, Ltd., and the Wellcome Laboratories. Research progress reports were circulated among these members, and in 1942 the importance of the work caused the Ministry of Supply to organize a general commission to deal with the various aspects of the production of penicillin—the Penicillin Producers Conference. This group included at times members of the above group and representatives of Imperial Chemical Industries, Ltd. (Alkali and Pharmacetutical Divisions), Imperial College of Science, Manchester University, the National Institute for Medical Research, and several laboratories of Oxford University (41).

The difficulties of expanding production of penicillin during the war emergency in Great Britain prompted solicitation of assistance from laboratories in the United States. During the early part of 1941 investigations were started on a small scale at the laboratories of the Squibb Institute for Medical Research and Merck and Co., Inc. A visit by Drs. Florey and Heatley of the Oxford group to various laboratories in the United States during July–September, 1941, encouraged interest, and several other laboratories started to study the problems involved in the mycological production of penicillin, including those of the Fermentation Division of the Northern Regional Research Laboratory of the United States

Department of Agriculture, located in Peoria, Illinois. Here two important observations were made by Dr. A. J. Moyer and associates shortly after inauguration of the experimental program. These included the observation that addition of cornsteep liquor to the modified Czapek-Dox medium previously used resulted in increasing the production of penicillin five- to 12-fold, and that cultures of the penicillin-producing fungus would grow well and produce penicillin when cultured under submerged conditions in aerated vats (44). Somewhat later in that year, with the approval of the concerned departments of the Department of Justice, representatives of the Squibb Institute for Medical Research and Merck and Co., Inc., agreed to exchange information, and they were later joined in this "pool" by representatives of Chas. Pfizer and Co. A similar "pool" was formed subsequently by representatives of Abbott Laboratories, Inc.; Parke, Davis, and Co.; Eli Lilly and Co.; and the Upjohn Co. Several other laboratories also studied the problems associated with the production of penicillin, including those of Commercial Solvents Corp., Winthrop-Sterling Co., Schenley Laboratories, Inc., and Hoffmann-La Roche, Inc. In December, 1941, a meeting was held at which information was exchanged among those interested in the problem, and several sessions took place in the years following.

Production methods were explored during the next 18 months, and several producers started planning construction of new facilities and large-scale production units. This expansion was held in check until clinical data were available emphasizing the importance of penicillin as a chemotherapeutic agent, and, more important from a mycological production viewpoint, until studies on the chemistry of the penicillins indicated whether or not an economical method of chemical synthesis was to be expected. The studies on the chemistry of the penicillins were coordinated in 1943 by a section of the Office of Scientific Research and Development (ORSD), and, as related in the monograph (41), representatives of 19 laboratories in the United States and ten in Great Britain exchanged information periodically. The magnitude of the effort may be gauged from the observation that over 700 reports were exchanged in less than three years by these cooperating laboratories studying the chemistry of the penicillins. The requirements of the armed forces for penicillin caused production of it in May, 1943,

to become one of the projects of the Office of Production Research and Development (OPRD) of the War Production Board. At that time construction of 18 production plants in the United States and two in Canada was in progress or contemplated (44, 45, 66, 67), and it appeared that a biological process would be most efficient and than an economical chemical synthesis was somewhat remote. In addition the research laboratories of several other pharmaceutical manufacturers were investigating problems involved in the production of penicillin.

The OPRD assigned the problem of increasing penicillin production to Dr. A. L. Elder who served as coordinator from October, 1943, to March, 1944, and to Dr. L. A. Monroe who assisted Dr. Elder in addition to having other assignments and succeeded him in 1944. The services of advisory groups to the OPRD were located at Pennsylvania State College where recovery methods were studied, at the Massachusetts Institute of Technology where drying techniques were investigated, at the University of Wisconsin where fermentation problems were studied (182), and at the University of Minnesota, Stanford University, and the Laboratory of Biology, Carnegie Institution of Washington, where methods of isolation of new penicillin-producing cultures were investigated (44, 67). While most of these projects were only of a few months' duration, since construction of plants and other developments proceeded rapidly, a staff of over 200 were employed at one time or another during the existence of these advisory groups, and some 80 progress reports were issued to those laboratories studying the production of penicillin. Perhaps the outstanding accomplishment of this effort of the OPRD was the selection and recognition of a new culture of *Penicillium chrysogenum*, designated as X-1612, in the latter part of 1944 which resulted in doubling the production of penicillin in a matter of a few months and more than repaid the small financial investment in these advisory groups.

The first experiments at Oxford University (2, 36) utilized the surface method of culturing the fungus. Later experiments (44, 67, 42, 66, 54) indicated that the organism would grow well and produce penicillin when cultivated in submerged culture, when grown on moistened bran, or when grown in a "vinegar" type generator. While most of the early production in the United

States (1942–1943) was the result of surface culture method, and while two plants were started using the bran process, the submerged culture method appeared to be the most economical from the standpoint of material cost, labor involved in operation, and production of antibiotic per unit of time (these methods will be discussed later in detail), and it was eventually adopted in 1944 as the method for the production of penicillin.

Once the production plants had started operation, the process development, as is usual in such cases, was somewhat restricted. The most marked improvement in production yields came as result of the use of new cultures (45, 196, 197) and the observation by Dr. A. J. Moyer and others that addition of phenacetyl derivatives

TABLE II

PRODUCTION OF PENICILLIN IN THE UNITED STATES*

Year	Billion units
1943	21
1944	1,633
1945	7,052
1946	27,809
1947	41,426
1948	95,855
1949	133,229

*Drawn from data summarized in Chemicals and Drugs, a monthly publication of the U. S. Department of Commerce.

to growing cultures encouraged the formation of penicillin-G. Production rapidly increased, as indicated in Table II, and distribution of penicillin for chemotherapeutic purposes, which had been restricted by a directive of the War Production Board allocating 75% to 85% of the production in 1943 and 1944 to the armed forces, was practically unlimited by the end of 1945.

Most of the investment in construction of production plants and laboratory research in the United States was financed by the industrial companies involved. It is estimated (45) that less than one-third of the $25,000,000 spent in construction was financed through government loans. The first studies (36) at Oxford University were financed in part by grants from the Medical Research Council, the Nuffield Trust and the Rockefeller Foundation. The studies at the Northern Regional Research Laboratory

were done under contract with the OSRD, and the laboratory investigations started by the OPRD were supported in part by grants from the government and the companies interested in production of penicillin. Sales of penicillin products had an estimated value of $200,000,000 in 1948, and during the past six years the price of 100,000 units of penicillin has dropped from $20 (1943) to less than $0.10 (1949), with the purity of the product distributed increasing from about 25% to 98%.

While the above summary is mainly concerned with penicillin production in the United States, principally because this information is available to the author and because many of the major production developments occurred in these laboratories, considerable effort was expended in other countries, notably Great Britain. By the end of 1945 production in Great Britain was approaching 200 billion units per month; in Australia, 20 billion units per month; and small amounts were produced in Mexico (45). The wartime secrecy order tended to delay development of large-scale production in other countries, and, although some effort was expended by the German government investigating penicillin production by the surface culture process, a successful process was not in operation by the end of the war. After hostilities were concluded production plants were started in nearly all of the European countries, as indicated by reports in the technical press, making use of the processes developed in the United States and in many cases following laboratory studies concluded under wartime conditions. There are plants in 25 countries manufacturing penicillin (*227a*), with nine in Great Britain, five in Germany, three in Italy, two in France and large installations in the Netherlands, Denmark and Sweden. Approximately 25% of the penicillin produced in the United States during 1949 was exported (value $40,000,000); this market is rapidly being reduced as the foreign plants reach maximum production. A few references in the Russian literature indicate that investigations were in progress there during the war, and information given to accredited visitors to the United States laboratories from the U.S.S.R., but unfortunately no information appears to be available concerning penicillin production there. Some small plants were started in China during the war, based on processes used in the United States, but here again information on production is lacking. Since

the end of 1945 a rather sizable penicillin industry has been started in Japan, and a number of papers in the literature indicate that the processes used there resemble those utilized elesewhere.

LARGE-SCALE PRODUCTION OF PENICILLIN

A brief summary of the evolution of the methods used for penicillin production has been presented in the previous section. It can be seen that this development which took place over a several-year period involved a considerable amount of work, study and ingenuity before the methods used at present were organized and accepted. One of the factors complicating this development was that this was a new type of fermentation process, producing only traces of a relatively unstable product, a situation which had not previously confronted fermentation chemists. Also the situation was complicated by the overwhelming demand for this product, which meant that little time, effort or fermentation capacity could be easily spared for rather basic studies of the various problems involved in penicillin production by the mold.

It was under these rather unsettled conditions that methods suggested for use in producing penicillin were evaluated. In nearly all of the early (pre-1941) laboratory studies the cultures had been grown in small flasks where the mycelium formed on the surface of the liquid, the so-called surface culture method. Expansion of this method to large scale operation would necessitate considerable investment in equipment as well as a high operation cost occasioned by the large labor force required to operate such units. Modifications of this surface culture method, suggested and tested on a comparatively large scale, included growing the cultures on moistened bran or grain, and growing them in towers similar to those used in the production of vinegar. At the same time considerable thought was given to the use of the submerged culture method for growing fungi, a technique which had been successfully involved in the production of gluconic acid. This latter method had the economic advantage that investment costs would be less than for the other culture methods, that the operational costs of these units would be lower, and, as will be discussed later, that the production from a relatively small unit would probably be equal to that from a large surface culture unit (bottle plant). However, the submerged culture method had not

been applied to the production of penicillin when these considerations were being made, and considerable doubt existed during the first studies that this method could be easily adapted to this fermentation.

As has been previously mentioned, this period of development extended from 1941 to 1943, and during this time several surface culture units, using bottles of the bran process, were constructed and operated along with the submerged culture units (44, 66, 54). During this interval the demand for penicillin far exceeded the supply, and the allocations for general use were quickly exhausted (44, 45, 203). A number of reports appeared in the literature suggesting the use of dressings impregnated with mycelium or periodically wetting the dressing with metabolism solution from fermentations. Apparently these methods were tried in a limited number of cases (106) which under the circumstances could not have been treated with penicillin preparations. For the most part these procedures suggested growing the fungus on a gauze pad soaked with a nutrient medium, and applying the whole to the wound. It is surprising that success was obtained with such crude preparations (106, 156) and that more untoward symptoms were not encountered. It is possible that if many of these poorly controlled treatments had failed, public faith in the potentialities of penicillin as a chemotherapeutic agent would have been shaken, as the use of these "home-made" preparations was comparatively widespread (191).

While the following discussion refers only to methods and problems encountered in the mycological phases of penicillin production, it should be kept in mind that the problems encountered in the recovery phases of the production were just as numerous as in the fermentation phase, and the recovery methods developed were fully as important to the successful operation of the production units as the fermentation procedures, if not more so (32, 54, 97, 253). Very often changes in fermentation procedures which appeared to result in increased production of the antibiotic could not be immediately introduced into production practice, for they also caused many problems in recovery of the penicillin from broth.

GENERAL CONSIDERATIONS. Before studying the various procedures used for the production of penicillin in detail, it seems

advisable to consider some of the problems common to all of these methods. Some of these problems are the result of the chemical nature of penicillin, including its comparative instability in slightly acid or alkaline solution at low temperatures, its loss of antibiotic properties after exposure to comparatively mild oxidizing or reducing agents, and its instability after contact with solutions of salts of certain metallic elements (41). Such chemical instability had been rarely encountered previously, and many of the research groups studying penicillin chemistry or production methods had had little experience with such unstable compounds. While those studying the fermentation aspects of penicillin production were usually not concerned with some of these characteristics of penicillin, the ease with which production goals were met was often complicated by the efficiency of the recovery of the antibiotic, and if recovery procedures were unsatisfactory the fermentation unit had to produce more penicillin to meet the demand.

Two chemical characteristics which affected the mycological production of penicillin were instability in alkaline solution and instability to heat (16, 41). During fermentation the pH of the culture gradually rises, and if allowed to proceed too far it may reach 8.5 which may result in inactivation of much of the penicillin present. This may also occur while the fermented medium containing the penicillin is awaiting processing (134), and experience has shown that some care must be taken to prevent the fermentation from undergoing these changes. Inactivation of the penicillin formed during the fermentation by heat was a problem in the bran process, as oxidation of the carbohydrate constituents of the medium by the fungus was oftened accompanied by a rise in temperature. Temperature control in units where tanks or bottles were used was not difficult, and this factor was of limited importance. While penicillin may be produced by most cultures when incubated within the range of 18° to 31° C., best results apparently have been obtained when temperatures have been controlled between 23° and 25° C. (2, 227).

In addition to being subject to chemical inactivation, the penicillin molecule may be irreversibly changed by enzymatic activity which simultaneously results in loss of antibiotic activity. This enzyme has been called "penicillinase" (1, 81) and has been found in preparations from a large number of Gram-positive and

Gram-negative bacteria (7, 63, 64, 141, 140, 153, 239, 81), actinomycetes (249, 259), the yeasts *Debaromyces guilliermondi* and *Mycoderma valida* (259), and the fungus *Papulaspora* (259). Many of these organisms may contaminate the penicillin fermentations, since they occur commonly in air and water, and if such contamination occurs at least a portion of the penicillin present will be lost. Thus it is almost absolutely necessary to operate the fermentation units under conditions of complete asepsis, and when this has not been achieved some losses have been noted. A number of antiseptic compounds have been added to fermentations in an effort to reduce the spread or growth of the contaminating organisms without affecting the production of penicillin (126), and addition of borax was effective in some instances. While this may be added as a prophylactic measure, and it is possible to have contaminants growing in the fermentation which will not inactivate the penicillin (259), there does not appear to be any satisfactory substitute for complete asepsis in fermentation operations (217).

The above discussion illustrates the need for adequate methods for sterilizing the fermentation units. The fermentation medium has usually been sterilized by heat treatment to kill contaminating organisms (54, 97, 227) by procedures similar to those used in other branches of the fermentation industry (113). Use of high pressure steam has found favor in the sterilization of the air filters, the fermenters and other equipment. Inasmuch as these fungi are aerobic organisms, they require large amounts of air for growth, and methods for sterilization of tremendous quantities of air were developed in connection with the submerged culture process. This was successfully accomplished by filtration through cotton, glass wool or other adsorbents packed tightly in steamable jacketed units (99, 182, 203, 226), by heat treatment (223) or by combinations of these and other procedures (217), as will be mentioned below in discussing the various processes.

While a detailed discussion of the disposal of wastes resulting from the mycological operations in the production of penicillin is outside the scope of this review, this is a rather important problem. Approximately 500 grams of medium ingredients (solids) are required at present for the production of three grams of penicillin, and more than three-fourths of these solids eventually have to be

disposed of as waste products. Some of these wastes, notably the mycelium, may be dried and utilized as animal or chicken feed where they act as sources of vitamins and proteins in the diet (129, 232, 258). Others may be disposed of by the treatment given to industrial sewage (129). This latter processing is complicated by the character of the material which has a high biochemical oxygen demand and imposes a severe strain on the ordinary disposal system. While there are no reports available indicating that the production units have been forced to close because of disposal problems, there is some evidence that as the selling price of penicillin fell the production cost declined at a slower rate, and in many cases waste disposal cost was an important consideration in determining whether a production unit was operating at a profit.

THE SURFACE CULTURE PROCESSES. In the experiments reported by Fleming (70) and Clutterbuck et al. (43) the cultures were grown on the surface of media in grass flasks of laboratory size. While this method is commonly used for the study of fungi in the laboratory, the shapes of the glass flasks are usually not well adapted for large-scale work, requiring much more space than is absolutely necessary, especially if conical in form; furthermore these flasks are usually rather fragile and subject to breakage after only slight jarring. The Oxford group (2) designed and used a rectangular parallelepiped-shaped porcelain-ware unit, equipped with a spout for addition and removal of media, which was placed in movable wire racks. This facilitated mechanical handling of large numbers of units during certain operations, including sterilization of media, incubation of inoculated units and harvesting of the fermented media.

While these porcelain-ware units had many advantages over Erlenmeyer or Fernbach flasks, they were not so widely used as the somewhat less desirable (from the standpoint of shape) glass milk bottles which were available and could withstand moderately rough physical treatment. Both the round and rectangular base units were used in penicillin production plants with the sizes varying from one pint to two quarts, depending on the manufacturer. Some pilot-plant studies were done with Pvitsky and Glaxo flasks (41*a*) as culture receptacles, although these flasks were not too

well adapted to mechanical handling. Some of the methods used in a production unit have been described by Ainsworth et al. (3) and illustrate a few of the problems involved:

"Pint milk bottles were charged with production medium at a ratio of 200 ml per bottle by means of an automatic bottle-washing and filling machine. After filling, the bottles were plugged by hand with non-absorbent cotton-wool, fitted with loose-fitting aluminum caps, and arranged in twelves in wire baskets to facilitate handling. The aluminum caps had been designed to permit a similar passage of air to that allowed by the cotton-wool plugs. When a closer fitting cap was tried it was found that the diminished aeration gave substantially lower titres".

"The medium was very suitable for bacterial growth, and it was found necessary to plug as well as cap the bottles previous to inoculation because most contamination was found to occur during the cooling, after autoclaving. Sterilization of the medium was effected by autoclaving at 2–5 lbs. per sq. in. for 30 min. This was not entirely satisfactory in all cases and some contamination by a *Bacillus subtilis*-like organism occurred in uninoculated, plugged and capped bottles. Autoclaving for 15 min. at 15 lbs. per sq. in. resulted in reduction in contamination, but also affected the medium. After autoclaving, the baskets of hot bottles were packed on to monorail carriers, which supported the baskets in an almost horizontal position, and passed through a cooling tunnel in which the temperature of the bottles was brought down to that of the incubators by a blast of air. The carriers were then moved to the spray-gun room".

"The spray-guns used to inoculate the production medium were Aerograph guns (type MP). Each gun was fitted with a cotton-wool air filter and a one-liter canister containing a filter of 50 per in. mesh wire gauze and connected to the gun by 6 ft. of rubber tubing. The nozzle of the gun was covered with a cap of cotton-wool which was tied in place and the whole gun put into a small calico bag. The canister head was covered with a square of cotton-cloth which was tied down around the canister. The gun and canister were then carefully packed into a specially constructed galvanized iron box and the whole sterilized by autoclaving for 1 hour at 15 lbs. per sq. in.".

"The sterilized guns were taken to the sterile room where the canisters were filled with inoculum and from there to the spray gun room where the nipple of the air filter was attached to the air line (compressed air at 5 lbs. per sq. in.), the canister being suspended 2–3 ft. above the bottles to be inoculated. The gun was removed from the bag and the nozzle flamed. The operator removed the metal cap from the bottle to be inoculated and discarded the cotton plug. The mouth of the bottle and nozzle were flamed. Inoculum was injected into the bottle as a fine spray for eight seconds. The mouth of the bottle was again flamed and the cap replaced. It was found that 192 bottles could be inoculated from one canister containing about 900 ml of inoculum by one person in 20 min.".

"The inoculated medium was incubated in two large incubators, each 165 ft. by 14 ft. and capable of holding four rows of 33 carriers. Harvesting was effected in a mechanical harvester in which bottles were inserted and drained of their contents for 3–4 minutes before being transferred to the input end of the bottle-washing and filling machine".

It may be seen from the above rather detailed description that operation of such a unit involved considerable organization, and even with the help of many mechanical devices, required much hand labor and was thus quite expensive to operate. Nevertheless, units such as this were in operation from 1940–1945 (182) and supplied most of the penicillin produced during the first part of the period. Previous studies on the production of gluconic acid (141), lipids, (181, 246) and citric acid (245) had shown that fungi could be cultured on the surface of media contained in large pans with surface areas of one to two yds.). These trays could be arranged as individual units or interconnected. Parker (174) has reported on some of the problems encountered in maintaining sterility when these units were used for production of penicillin:

"Some idea of the magnitude of the problem can be gained from the picture of a factory area of five acres in which ½ acre of mycelial felt was growing in trays at any one time and over which *ca.* 30,000 cu. ft. of air passed per hour. Some 20 vessels connected by thousands of feet of pipework and hundreds of valves were used in the enclosed transfer and incubation of several thousands of gallons of medium. All this equipment was maintained in pure culture at the hands of unskilled operators rather than trained microbiologists. The fundamental principles which allowed successful operation of these units included: a) the technique used for preparation of starter cultures in the laboratory must ensure completely the exclusion of contaminants; b) the plant must be so constructed that all parts of it can be sterilized and that it encloses the culture from contamination; c) the air supplied to the plant cultures must be sterile; d) operation of the plant must be specified in such detail that relatively unskilled personnel can perform this in strict accordance with the simple sequence of actions laid down; e) every stage of the system of culture must be carefully checked by examination of samples for the presence of living microorganisms other than the organism being cultured".

"Re-use" of mycelium was possible in this plant. In this procedure, which has been resorted to in other fungal fermentations, the mycelial pad is grown as usual, and at the end of the incubation period the medium below the pad is carefully withdrawn and replaced with fresh medium. The time required for fermentation of

the new medium is less than for the first batch, as the mycelium is already developed. This procedure was used by the Oxford group (2) who were able to replace the medium 14 times without reduction in penicillin production, and has also been depended upon by others (162) who applied this technique in surface culture and submerged culture operations.

Tremendous numbers of spores are needed to inoculate the bottles as described above. For laboratory purposes these may be produced by growing the cultures on surfaces of agar slants or similar semi-solid media. Among the agar media that have been used is one containing glycerol, molasses, cornsteep liquor, peptone and inorganic salts (159), a honey-peptone mixture (225), a beet-molasses agar (178) and other combinations (89, 198). While nearly all cultures tested grow well vegetatively on these media, such growth may not be accompanied by sporulation, and very often variation in the concentration of certain ingredients in the medium, e.g., sodium chloride, or change in the incubation conditions will be required to obtain large numbers of spores with certain cultures (92). In a study of factors affecting the production of spores by *Penicillium notatum* (89) it appeared that there was some association between the depth of the agar medium and number of spores produced per unit area. An increase in the concentration of metabolites above the normal level did not greatly increase the yield of spores, and some evidence was obtained which was interpreted to indicate that the production of spores was limited by accumulation of toxic substances in the medium.

One practice which received study in production units was to cultivate the mold on a finely divided organic material. Rice bran, bread crumbs, ground potatoes, oatmeal, rye, wheat and rice have been used (88*a*, 99). Some success was noted (158) with a mixture of barley perlings, red dog flour, wheat bran, middlings and oats. These substances are often badly contaminated with spore-forming bacteria, and as the heat transfer characteristics of these materials is low, it is often difficult to obtain complete asepsis by the commonly used sterilization procedures.

After these agar or organic media have been inoculated and the cultures have grown and sporulated, sterile water or mixtures of water and surface active agents are usually introduced into the

sporulation vessels, and the spores shaken or scraped off the vegetative growth. The suspension formed may be used immediately to inoculate the fermentation units, or may be stored for a short period (at 5° C. or lower). It should be kept in mind that while it is possible to obtain large numbers of spores by these methods, not all of these spores will be viable (225), and apparently, as will be discussed later, not all cultures derived from single spores have the same penicillin-producing capacity or characteristics. While most of the spores remain viable for some time, common practice (92) has been to use spores which are less than one month old. Stock cultures have been prepared by lyophilization of spore suspensions, drying of spores on soil, or other techniques, and in general some degree of success has been obtained in keeping the cultures in a viable form.

In the production of acetic acid or vinegar by oxidation of alcoholic solutions by *Acetobacter* sp., an aerobic process has been used, and at one time it was thought that a similar fermentation apparatus could be utilized for production of penicillin (67). In this process for production of acetic acid (175) tall vats filled with beechwood shavings as a contact surface and a support for the fermentation are employed in series. The medium is introduced at the top of the first vat and trickles down over the shavings countercurrent to the natural convection of air stirred up by the heat of the oxidation reactions. Air for the oxidation is supplied through a perforated false bottom. The liquor leaving the bottom of the tank is introduced at the top of the next vat. A unit of this type was operated successfully on a pilot-plant scale for penicillin production without contamination (42), but this process was not widely used because of difficulties in operation, including temperature control, air sterilization and prevention of contamination. These same objections were encountered when adaptation of similar processes (31, 145) used in the production of citric acid were tried for the production of penicillin, and served to emphasize that penicillin production differs from the production of other substances by mycological processes.

A rather ingenious device (228) in which the mold is grown on surfaces of liquid below the surface of the liquid in the whole

unit has been described. Air is introduced into the unit and trapped in pockets below the surface of the liquid. The fungus forms on the liquid-air interface and grows. The liquid is then circulated through the unit, and as penicillin is produced the liquid is harvested and fresh medium is added. This unit was tried on a laboratory scale and worked successfully, but has obvious limitations which eliminate it as a large-scale production process.

In one of the processes for the production of amylases by fungi, the organisms have been grown on the surface of bran (238). When aerated vigorously the cultures grow rapidly, and a number of strains of *P. notatum* have been found to grow well and produce penicillin under these conditions (220). Other grains, including moistened oats, have also been used (148). In one modification of the process the bran mixture was inoculated with the mold and spread in thin layers on trays over which sterile air was passed (54). The growth of the mold was allowed to proceed for the desired period, and then the penicillin present was leached and pressed from the mixture of bran and mycelium. Under these fermentation conditions contaminations were a serious problem, and also it was difficult to prevent local overheating. These difficulties were overcome in part by placing the bran medium in long horizontal drums which were provided with baffles, a means of rotation and cooling jackets. After sterilization the contents were cooled and inoculated. Fermentation proceeded during rotation of the drums, and oxygen was supplied by introducing sterile air at the trunions. Even with these modifications contamination was still a serious problem, and eventually the process was abandoned in favor of the deep-tank process.

THE SUBMERGED CULTURE PROCESS. During the past 15 years considerable interest has been evident in the growth of fungi in submerged culture, e.g., the organism is grown in media which are aerated and agitated so that the mycelium spreads throughout the liquid. Among the first to use this technique of growing fungi for laboratory studies were Kluyver and Perquin (115) who grew their cultures in shaken Erlenmeyer flasks. Shortly after the results of their experiments became known this technique or modifications of it (growing cultures in aerated bottles or aerated revolving drums) (102, 241) were used in studying the production of gluconic acid by *Penicillium chrysogenum* (157) and *Aspergillus niger* (248). These earlier studies suggested to investigators at

the Northern Regional Research Laboratories (163) that it might be possible to produce penicillin by growing the cultures in this manner, and investigations were in progress with cultures of *P. notatum* in August 1941 and later with the related *P. chrysogenum*.

In the laboratory investigations the equipment used included shaken Erlenmeyer flasks, revolving drum fermenters, small aerated vats and stirred jar fermenters. (Pictures of several types of shakers and stirred jar fermenters are found in citations 74, 182, 203*a*). Many of these pieces of equipment were fabricated by those working on the fermentation problems or by machine shops in the vicinity of the laboratories, and it is not surprising that some variation in design and operation occurred. The speed of the reciprocating shakers varied among the different laboratories from 80 to 250 cycles per minute, and that of rotary shakers from 125 to 375 revolutions per minute with an amplitude of one-half to four inches. In all cases the object of the shaking was to increase the amount of dissolved air in the liquid, and this shaking often resulted in doubling the oxygen content (224). When grown under these agitated conditions the fungus assumes many morphological shapes (30, 60*a*), most often resembling a suspension of paper pulp (60*a*).

These shaken flask fermentations permitted evaluation of a number of aspects of the fermentation, including the effect of medium composition on penicillin production, comparison of the penicillin-producing ability of cultures, and the testing of certain antifoam solutions and construction materials for toxicity effect on penicillin production (226, 227). However, the method is essentially a laboratory method, and the conditions inside the flasks certainly do not resemble physically those found in aerated tanks used in production units. While small flasks containing sintered glass aerators were perhaps more like these tank units in design, they could not be used in the laboratory, since the fungus grew through the sintered glass aerators of the carborundum stones and resulted in complete clogging. This led to use of aluminum tubing which was perforated with small holes (1/64 inch) and eventually to stainless steel units which were agitated and aerated in a manner resembling that which had been developed for use in production plants (23, 26–28, 135, 182, 203*a*, 74). These stirred jar units made possible a limited study of the effects of aeration and agitation rates on mold growth and penicillin production (23, 26, 28),

and penicillin production in these units often approached or surpassed that obtained in larger units. In recent studies a laboratory version of the Waldhof fermenter (25, 206, 207) has been tried, and it may indicate possible improvements of existing designs in production units. Some study has apparently indicated that recirculation units may be incorporated in fermenters for production of penicillin (65).

While operation of these stirred jar units is comparatively expensive from the standpoint of time involved and information gained from the study of experimental variables as compared with the shaken flask units, they are less expensive and more adaptable to changes than larger tank units. Most factors in the design of

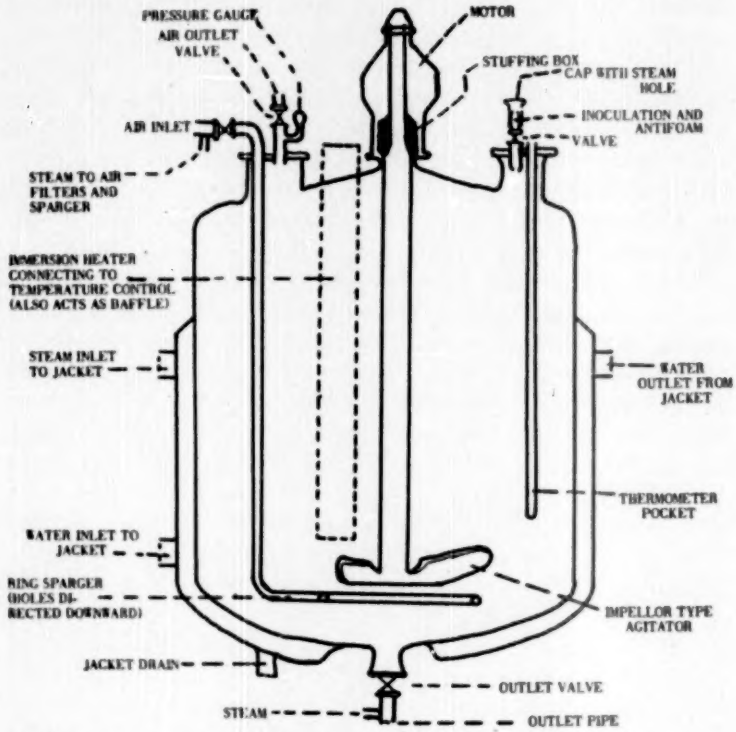


FIG. 2. Diagrammatic vertical section of pilot-plant apparatus for penicillin production. (Adapted from Gordon, *et al.* (97), reprinted with permission of Dr. T. White).

these large-scale production units have not been revealed, and apparently considerable variation exists in equipment design and operation with many types of agitators and aerating devices, metals and alloys finding some advocates among the different manufacturers. It is not surprising to hear that conditions which one manufacturer finds satisfactory will not work for another. Some of the variations may be seen in pictures of production units (32, 97, 45, 172, 185), but these reveal little of the details or operational procedures. The essential details of a pilot-plant unit are in Fig. 2, and include an air sparger, a water jacket or coils for temperature control, an agitator, sampling lines, antifoam addition mechanism, inoculation lines, transfer lines to other fermenters, cookers and recovery units similar to those in other fermentation equipment (113). Another pilot-plant unit has been described by Stefaniak et al. (226). Operating details for these pilot-plant units have been provided in the respective publications (97, 226, 74*a*, 73*a*, 190*a*). Apparently most of these units have been operated on a single-batch basis where the medium is fermented and harvested as a unit. However, some interest has been shown in operation of a continuous fermenter where medium is constantly added and withdrawn (215).

Sterilization of these units has introduced many new problems, and the complexicity of this operation may be judged by Parker's remarks (174):

"In designing and constructing the penicillin plant we have followed a number of well defined principles which were established very early in our experience of large-scale working. These principles are as follows:

a) No direct connection should be made permanently between non-sterile and sterile parts of the system.

b) Welded constructions should be used where possible and convenient.

c) Where joints have to be used, these should be of a high quality finish, employing rubber as the sealing material.

d) The type of valve used on sterile process and service lines should be carefully selected so that it can be easily sterilized and serviced when necessary (it should be capable of maintaining pure culture conditions for the duration of the fermentation cycle).

e) After sterilization, all parts of the system which are to be kept sterile should be kept under a positive pressure, either with sterile air or sterile liquor, during the cooling period and the fermentation period.

f) Each part of the system should be capable of independent sterilization without interfering with the operation of the rest of the plant.

g) Isolated danger spots which are difficult to sterilize at the start of a batch, should be provided with pressure stem connections for continuous or intermittant use".

Application of these principles usually will insure uncontaminated fermentations. A number of methods used for the sterilization of the air necessary in the fermentations have included filtration (97, 217) and heat (223).

Preparation of inoculum for submerged culture fermentations may follow the procedures of the surface culture method, namely, growing the cultures on a medium which results in profuse sporulation and inoculating the fermentation medium with a suspension of these spores. This may be done stepwise (99) : a loop of spore suspension is taken from a master stock culture kept on soil and transferred to an agar slant and incubated for five days at 25° (several types of media have been used) ; five ml. of distilled water are then added and the tube is agitated to remove the spores. The spore suspension so obtained is used to inoculate one 20-oz. Roux bottle, three plates of modified Czapek's agar and two rye grain bottles. The plates of Czapek's agar permit checking the suspension for the presence of contaminants, and they and the Roux bottle also serve to check the culture for the presence of morphological variants. Changes accompanying morphological variation may also affect penicillin production, as has been noted in at least one plant (99), and explained why penicillin production dropped markedly.

The two rye grain flasks are used as sources of spores for inoculation of a ten-liter aspirator bottle (99). The spores germinate in this aerated bottle, and the vegetative growth may then be transferred to inoculate large volumes of medium contained in fermenters. In some cases under extreme circumstances rather large fermenters (ca. 10,000 gallons capacity) have been inoculated directly with grain spore suspensions. This eventually resulted in good fermentations, but considerable time was taken while the culture was growing, and very expensive equipment was not being used for penicillin production during this time. The use of a number of intermediate vegetative stages provides a different medium for growth of the inoculum than is used for the growth of

the fermentation. The reports at hand (92, 226, 88a) indicate that most *Penicillium* cultures grow well on media containing glucose, sucrose, starch and dextrin but that penicillin production is low on such media. On the other hand, as will be discussed later, less growth and more penicillin production are obtained on media containing lactose, and apparently these media containing glucose or glucose derivatives are used in the inoculum stages. However, one report (179) indicates that there must be some caution in selection of the inoculum medium, and results as summarized in Table III may occur if care is not exercised in choice of medium. The culture may be grown through several vegetative stages with increase in volume from ten to 1000 gallons without further treatment, or it may be homogenized at the first stage to give it a faster start (211, 240).

In the above description the cultures were grown to the sporulation stage on semi-solid or solid media. Cultures have also been observed to form spores when growing submerged in several types of media (8, 94, 183). Spore counts of 5×10^8 viable spores per ml. have been reproducibly obtained on an aqueous medium containing glycerol, molasses, sodium cloride, peptone, magnesium sulfate, iron tartrate, copper sulfate and potassium phosphate. These spores are said to be just as satisfactory for penicillin production as those obtained by culturing the fungus on solid media.

As each pilot-plant unit and large-scale fermentation unit apparently has its own peculiarities with regard to optimum rates of aeration and agitation, extended discussion of these items is not advisable here. In addition there is evidence that different cultures require different aeration rates (23, 226), and it seems that each fermentation unit must be studied by almost trial and error procedures to determine the optimum aeration and agitation rates best suited for the cultures (255a). Some idea of the effectiveness of the aeration may be determined by measurement of the carbon dioxide content of the exhaust gas (120, 221). In most studies where an aeration rate of one volume of air per volume of medium per minute has been found optimal, as measured

TABLE III

EFFECT OF INOCULUM MEDIUM COMPOSITION ON PENICILLIN PRODUCTION

Inoculum medium carbohydrate*	Fermentation medium**	Maximum penicillin production units/ml
none	Lactose-cottonseed meal	910
	Lactose-peanut oil meal	765
	Mannitol-peanut oil meal	765
Glucose	Lactose-cottonseed meal	850
	Lactose-peanut oil meal	735
	Mannitol-peanut oil meal	800
Lactose	Lactose-cottonseed meal	960
	Lactose-peanut oil meal	695
	Mannitol-peanut oil meal	700
Mannitol	Mannitol-peanut oil meal	725
	Lactose-cottonseed meal	835
	Lactose-peanut oil meal	760
Starch	Lactose-cottonseed meal	950
Inulin	Lactose-cottonseed meal	1055
Sorbose	Lactose-peanut oil meal	880
	Lactose-Cottonseed meal	825
Xylose	Lactose-peanut oil meal	660
	Mannitol-peanut oil meal	860

*Inoculum medium composition (grams per liter): carbohydrate, 20 g.; nitrogenous material (as in fermentation medium), 20 g.; tap water q.s. 1 liter.

**Fermentation medium composition (grams per liter): lactose, 30 g., or mannitol, 30 g., as indicated; cottonseed meal, 40 g., or peanut oil meal, 40 g., as indicated; calcium carbonate, 10 g.; soybean oil, 3.2 ml; tap water to 1 liter.

Inoculum: 5% of 48 hour vegetative growth of *Penicillium chrysogenum* Wis. Q176 grown on the indicated medium.

Abstracted from Perlman (179).

by penicillin yields, the carbon dioxide content of the exhaust gas has not exceeded 1.5%, indicating that nearly 90% of the oxygen supplied has not been utilized by the mold for oxidation of the carbohydrate. In certain studies (23) a straight line relationship has been postulated for the log of the maximum penicillin titer and the log of the respiration rate, but this relationship has not been apparent in studies in other laboratories and may vary considerably with the type of aeration equipment.

During the vigorous aeration and agitation of these fermentations large quantities of foam are formed, and, unless dispersed, considerable quantities of the contents of the fermentation vessels may froth out the air escape line. A number of anti-foam materials have been tested for ability to break this slightly acid foam, and of those examined (97, 139, 226, 227, 190*a*), vegetable or animal oils, namely, corn oil (32) or lard oil (226, 227), often containing other surface active ingredients (119, 25, 140,), have found widespread use. These are apparently non-toxic (227) to the fungus and in some cases appear to promote penicillin production, perhaps by increasing aeration efficiency (190*a*, 224) rather than reducing the destruction of the penicillin (138), or by supplying unsaturated fatty acids which may be required for rapid growth of the mold and for penicillin production (139, 19*a*).

ANALYSIS OF FERMENTATION SAMPLES FOR PENICILLIN CONTENT

The methods of the determining penicillin content of samples taken from fermentations have been of considerable importance in the mycological production of penicillin. A short consideration of these methods is included here to afford a perspective of the work that has been done, and how it has affected development of the penicillin industry. The various chemical and microbiological methods in operation prior to 1946 have been extensively reviewed in the monograph on the chemistry of the penicillins (41) and will not be developed here in detail.

Fleming (70) used an agar plate method in which a dilution of the penicillin-containing solution was added to part of the agar in the Petri dish, and the surface of the agar was streaked with susceptible microorganisms. The highest dilution at which inhibition of the test bacteria was noted was taken as indication of the potency of the preparation. Variations of this agar dilution method have been widely used since in the study of antibiotic substances (244). In the early studies at Oxford University (2) a modification of this method was devised by Dr. N. G. Heatley (101) and has since been adopted as the standard procedure. In this modification small cylinders were placed on the surface of an agar layer previously inoculated with *Staphylococcus aureus*, and solutions containing penicillin were placed in these cylinders and allowed to diffuse through the agar. Zones of inhibition of the

S. aureus growth caused by the antibiotic were measured after incubation of the plates for 14 to 18 hours. The diameter of the zone was found to be practically a logarithmic function of the concentration of antibiotic present, and comparison with size of zones obtained in the same period with standard preparations made it possible to estimate the potency of unknown solutions. This "cup-test" method, as it has been called, has been studied in detail, and many of the factors affecting the procedure have been investigated, including the effect of the pH of the samples, presence of ionized salts in the sample, composition of the agar medium (41, 101, 78, 80, 212) and inherent errors of the method (29). The difficulties in standardization have been well illustrated in the comparative studies participated in by many industrial laboratories (130), which indicated very wide variations in the analysis of impure samples.

A number of modifications of the method devised by Heatley (2, 101) have been tried at various times. These include small discs of blotting paper instead of the cylinders (41, 216, 241), and use of holes in the agar cut with an implement, e. g., cork borer, as reservoirs for the penicillin solution (29). Large dishes on which as many as 90 cylinders were placed have been substituted for the relatively small Petri dishes whose capacity is five or six cylinders (8, 29, 41), and a large number of mechanical devices have been assembled to increase the number of assays that can be done by a limited number of operators. A few of these mechanical devices have included those for quickly adding agar to plates (214), devices for placing the cylinders on the agar (40, 201) and devices for measuring the size of the zones of inhibition (171).

A large number of alternative methods to that of Heatley have been proposed at various times. In several of these inhibition of the test organism in broth culture after addition of the antibiotic solution has been measured. This may be done by turbidimetry (41, 75, 77, 12, 146) or indirectly by measurement of some other property of the culture besides growth (98). However, the presence of glucose-aerodehydrogenase (notatin) (52, 53, 131, 132, 133, 204) in the samples may affect the growth of the test organism in liquid culture if glucose is present in the medium. This enzyme does not diffuse rapidly through agar and has no appre-

ciable effect on the determination of penicillin by the cup-test method. The presence of other substances in the samples to be assayed, e. g., fatty acids, will also affect the turbidimetric test (41), and for practical purposes the cup-test has been more widespread than any other method.

Substitution of other organisms for the cultures of *Staphylococcus aureus* and *S. albus* used by Heatley and others has been tried without much success (41, 77). When this substitution was first made the results with one test organism varied greatly from those achieved with other organisms. This was later explained by the observations that the *Penicillium* cultures under study were producing more than one type of penicillin at one time (41), and the bacteria reacted differently to the different types present in the samples. For example, penicillin–K is much more active on a weight basis than penicillin–X when *S. aureus* is the assay organism, as indicated in Table I, while the relationship is reversed when *Bacillus subtilis* is the assay organism (41). Thus, while a spore-forming organism is much easier to use as an assay organism than *S. aureus* (216, 76), it is often difficult to interpret the results (213), and since penicillin has been defined in terms of the inhibition of *S. aureus* (41), it is probably better to use the latter organism as the assay organism.

This variation in response of different organisms to the different penicillins has been relied upon as a method of estimating the proportions of the different penicillins in the samples (41, 104, 213). These methods are subject to many errors, especially if the number of penicillins in the samples under study is not known. It has also been shown that the effects of the penicillins on the test organisms are not additive (60), which limits the use of these microbiological differential methods to qualitative instead of quantitative purposes.

Other methods for determination of the various penicillins in mixtures have combined a physical separation with the microbiological assay. The mixtures have been separated by countercurrent extraction (56, 57), aqueous chromatography (235, 19*a*) or paper-strip chromatography (69, 96, 123, 124, 254), and the fractions collected have been assayed microbiologically. Study of these procedures has indicated that very small quantities of penicillin are required, about one-half unit, for complete analysis,

and a rather accurate quantitative as well as qualitative estimation may be made of all the penicillins present. However, the procedures are time-consuming, and periods of 24 to 48 hours are required before the results of the analyses are available.

Several chemical methods have been proposed for the quantitative determination of penicillins, but many of these are applicable only to purified samples. Total penicillin may be determined (74) or specific analyses may be made for penicillin–G (21). Inasmuch as the main commercial interest at present is in the production of penicillin-G, these chemical methods may be quite satisfactory, and knowledge of the identity and quantities of the other penicillins in the samples may not be desired or necessary.

The above discussion has been included in this review because it is felt that one of the most important aspects of penicillin production is an understanding of the importance of the part played by microbiological assays. If the penicillin content of samples from fermentations are not accurately determined, the conclusions regarding the value of certain media, choice of cultures or certain operations may not be easily made, and much effort will be (as has been) wasted in repeating critical experiments and pondering over apparent intangibles. As can be seen from the above discussion, a number of methods have been used over the past few years, and these have changed with time as a result of better understanding of the constitution of the penicillins. Thus interpretations of the data collected in some of the early studies (pre-1945) and published in papers appearing during that period should be different than originally proposed. In the following discussion an effort will be made to reevaluate these studies in the light of the more recent developments.

SELECTION OF CULTURES FOR PENICILLIN PRODUCTION

HISTORICAL DEVELOPMENT. All of the early studies (pre-1941) on penicillin production were made with the original Fleming culture, and during that period the hypothesis seems to have developed that this particular culture was unique in its ability to produce penicillin. This theory did not appear reasonable in view of experience with other mold fermentations, including those producing citric acid, gluconic acid, lactic acid, sterols and oxalic acid, where considerable variation was noted in the production of these

products by different strains of the same genus. The Fleming culture also appeared to throw off variants which were less desirable as penicillin producers (202) and perhaps accounted for some of the low yields noted (2).

Later studies (1942) at the Squibb Institute for Medical Research showed that a monospore isolate from the Fleming culture produced significantly more penicillin than the parent culture in repeated experiments. Further application of selection technique resulted in the isolation at the Northern Regional Research Laboratory of a rather stable culture from this Squibb culture which was designated as NRRL 1249.B21 and found use in the surface culture process. Most of the penicillin prepared for the earliest chemical and therapeutic studies was produced by this culture. As might be expected from its background as a natural variant, it was likely to "throw off" variants. A study of these variants (79, 250, 192) indicated that many produced less penicillin than the parent culture. Morphological variation was noted in two directions, namely, increased sporulation and pigment production, suggestive of the original Fleming culture; and reduced exudate formation and decreased sporulation in the direction of sterile colonies. One study indicated that mass spore transfers did not maintain good production of penicillin by this strain, the fungus apparently undergoing rapid natural variation with the production of mutants which usually sporulate both more rapidly and more heavily than the parent types (250). Low-yielding mutants derived from this high-yielding strain were much more stable than the parent, and attempts to build up penicillin yield of one of these low-yielding mutants by continuous selection were unsuccessful.

At the same time that studies were in progress with strains derived from the Fleming culture, other investigations were started, studying the production of penicillin by other cultures of Penicillia. A group of cultures belonging to the *Penicillium notatum-chrysogenum* group were tested for penicillin production when grown in surface and submerged culture, and nearly all of the 35 examined were found to possess some antibiotic activity (159). Consideration of this rather extensive study as well as others (85, 243, 219) on cultures of the *P. notatum-chrysogenum* group suggested that *a*) the capacity to produce penicillin as a

metabolic product represented a group rather than a strain characteristic, *b*) different members of the group varied greatly in their capacity to produce penicillin, and *c*) particular strains were especially suited for certain production methods.

One of the *P. notatum* cultures, designated as NRRL 832, was widely used for penicillin production by the submerged culture method during the period 1942-1944. Attempts to isolate higher-yielding substrains from this culture by various techniques failed to produce any markedly superior penicillin-producing cultures (110) but did reveal some striking cultural (192, 221) and physiological variations (135).

A survey of other strains of *P. chrysogenum* for penicillin-producing ability when grown under submerged conditions was made in a number of laboratories, including the Northern Regional Research Laboratory (193, 195) and the Department of Plant Pathology, University of Minnesota (44, 182). A large number of cultures belonging to this genus were isolated and found to produce penicillin under these conditions. One of those isolated from a molded melon and designated as NRRL 1951 produced approximately as much penicillin in the first studies as did NRRL 832, and substrains from this culture were found to yield nearly twice as much penicillin as the parent. One of these, NRRL 1951.B25, was selected for further study, and although continued investigation of natural variants of this culture failed to reveal any subculture capable of producing higher penicillin yields than the parent stock, the widest variety of cultural types was separated out (45, 193).

This culture, NRRL 1951. B25, found wide use in the industry during the period 1944-45, and at least part of the increase in penicillin production during this period (Table II) was due to the change in cultures. When spores of this culture were subjected to x-ray and then the survivors studied for penicillin production, some increase in penicillin yield was noted with one culture designated as X-1612 (58, 121). This culture produced approximately twice as much penicillin as its parent on a cornsteep-lactose medium not supplemented with a penicillin-G precursor (121, 227, 92), and found immediate use in the industry. At this time interest centered on producing penicillin-G, since chemotherapeutic studies had suggested that this type of penicillin is

as desirable as any and perhaps more effective than most as well as being more stable in aqueous solutions (17, 41). Study of the types of penicillin produced by culture X-1612 indicated that large amounts of penicillin-K were being produced on certain media, although the analytical methods used at that time were admittedly somewhat inaccurate. Supplementation of the media with a penicillin-G precursor resulted in an increase in the penicillin-G content of the penicillin formed and usually an increase in the total amount of penicillin produced (85, 190, 104).

While these studies with X-1612 were underway, investigators at the University of Wisconsin were studying the possibilities of increasing penicillin production by substrains of this culture through treatment of the parent strain with ultra-violet light. During the spring of 1945 a culture was isolated which produced considerably more penicillin than did the parent (6, 85), and when tested on various media it was found to be a consistently higher penicillin producer than the X-1612 culture. This culture, Wis. Q_{176} as it is now known, when grown on synthetic media produces very small amounts of penicillin-G and large amounts of the aliphatic type penicillins (123, 254). When grown on media supplemented with the phenylacetyl type compounds it produces large amounts of penicillin-G and small amounts of the aliphatic type penicillins (123, 218, 7). It has been widely used industrially since its first tests in October, 1945 (85). Efforts to induce mutations in this culture have been in progress during the past few years, and some success has been noted, with several cultures reported to produce several times as much penicillin as this relatively famous culture (7, 25, 88, 200).

The above discussion has indicated very briefly what success was noted in increasing the production of penicillin by culture selection. A few of the cultures used industrially and some of their characteristics are summarized in Table IV which may aid in making a comparison of the ability of these cultures to produce penicillin when grown in submerged culture. Indeed perhaps the most marked increases in penicillin production were noted after introduction of new cultures, and at times it seemed that perhaps the easiest way to increase penicillin production was to find a new culture. This idea found favor with certain of those operating some of the penicillin plants, but placed a tremendous

TABLE IV

CULTURES USED FOR THE PRODUCTION OF PENICILLIN

Designation of cultures	Source (parent culture)	Method of Selection	Penicillin production* on cornsteep-lactose medium: Not supplemented with penicillin G precursor	Supplemented with penicillin G precursor	Ref.
			units/ml	units/ml	
NRRL 1249.B21	Squibb 4	natural variant	143	194	161
NRRL 832	T. C. B-69		95	141	161
NRRL 1951	...	new isolate	100	...	195
NRRL 1951.B25	NRRL 1951	natural variant	175	250	195
X-1612	NRRL 1951.B25	x-ray	200	350	85
Wis. Q_{176}	X-1612	U-V ray	650	1,300	85
...	Wis. Q_{176}	U-V ray	1,100	3,050	88

*In laboratory shaken flask tests for all cultures except NRRL 1249.B21 where surface culture process was used.

responsibility on the mycologist working with the cultures. Literally thousands of cultures were tested before the isolation of Wis. Q_{176}, and it is probable that many thousands more were tested before new ones were developed from that parent. While this may be the first time that mycologists were accepted as important investigators in the fermentation industry, they had to work to produce their contribution, and often the odds against which they operated are not appreciated.

The above discussion has been concerned with the production of penicillin-G and with isolation of cultures particularly suited to the production of this type of penicillin. At one time there was a demand for penicillin-X, and a study was started at the Northern Regional Research Laboratory for production of this substance. Investigators at that laboratory were able to isolate a culture which produced large amounts of penicillin-X when grown under submerged conditions (194). If there is a demand for this type in the future perhaps this and other cultures derived from it will be used.

METHODS OF SELECTING HIGH-PRODUCING VARIANTS FROM PENICILLIN-PRODUCING CULTURES. As has been mentioned earlier in this discussion, studies with the Fleming culture suggested that some improvement in penicillin production could be obtained by study of the penicillin-producing ability of monospore isolates from this culture (192, 79, 197). These and other studies indicated that considerable variation was to be expected between different cultures selected in this manner (209, 209*a*), and, while one study suggested that stability of penicillin-producing ability was to be found when spores were used as sources of inoculum (79), another concluded that at least with culture NRRL 1249.B21 a vegetative transfer was to be desired if natural variation and consequent change (reduction in this case) in penicillin production was to be avoided (250). In studies with cultures used in submerged processes some improvement was noted in penicillin production when the production of a number of natural variants of culture NRRL 1951 were tested, and one of these, identified as NRRL 1951.B25, consistently produced more penicillin than the parent culture (192, 197). This approach has been used in trying to isolate improved cultures from Wisconsin Q_{176}, but no significant increases have been reported (200). Perhaps this is an indication that this latter culture is less likely to "throw off" variants, or it may be an indication that the variants produced are less noticeable than those produced by other cultures (7, 200).

The studies at Stanford University and the Carnegie Institution suggested that cultures producing more penicillin than the parent culture could be isolated from spore suspension that had received ultra-violet or x-ray treatment (58, 45, 197), and these two methods have been rather widely used (7, 200). This approach had been previously adopted by Hollaender (110, 111) in culture NRRL 832 with some success, but the results were not so striking as noted with the progeny of culture NRRL 1951, cultures X-1612 and Wisconsin Q_{176}; this earlier observation has often been overlooked. Further improvement was noted in a culture isolated when spores of Wisconsin Q_{176} were exposed to ultraviolet light for approximately one hour without stirring (88, 180). This culture, which produces more than two and one-half times as much penicillin as the parent, was also different from it in several physiological characteristics. Irradiation of it resulted in

isolation of somewhat better cultures in line with observations previously cited.

Treatment of spore suspensions with a number of chemical agents thought to induce mutations in fungi has also been tried. The agents tested include camphor (135, 208, 209), colchicine (135, 210), nitrogen mustard (200, 210) and chloral hydrate (135). These studies have resulted in cultures producing more or less penicillin than the parent culture, but the improvement has not been so striking as when irradiation has been used.

Exposure of spore suspensions to neutrons (100, 170) and inclusion of radio-active phosphorus in the medium (117) have also been tested as means of isolating new high-producing cultures. Here again, while improved cultures were noted the improvement was less than that noted with ultra-violet irradiation, and the latter method appears to be the method of choice at present.

PIGMENT PRODUCTION BY PENICILLIN-PRODUCING CULTURES. Many of the cultures of the *P. notatum-chrysogenum* group which produce penicillin also produce a number of yellow pigments (43, 79), and, although pure penicillin crystals are colorless, most of the relatively impure preparations distributed prior to 1946 had a decided yellow tinge which caught the attention of the public and was popularly associated with penicillin. The yellow pigments may be made colorless by reduction with mercury, or they may be removed by chemical treatments (41*a*, 45, 54), but these steps are quite expensive in that a significant quantity of penicillin may be lost during the processing. However, as the manufacturers of penicillin preparations improved their processes the color of the final product became lighter with increased purity, although the crystals often have a slight yellow tinge. When it was realized (41, 255) that pure penicillin would be colorless, search for penicillin-producing cultures which did not produce these pigments was intensified. Several of the cultures isolated from soil samples studied at the University of Minnesota gave significant quantities of penicillin and at the same time did not produce the yellow pigments. However, they were relatively poor penicillin producers and were not widely used, since the manufacturers apparently were not in a position to reduce production in order to produce a purer product.

Recently high penicillin-producing variants have been isolated from the Wisconsin Q_{176} culture which do not produce these yellow pigments when grown on agar or liquid media (7, 25). Further isolations from these cultures by various techniques have resulted in a number of cultures which do not display these pigments and at the same time produce more penicillin than the parent culture (7, 139) when grown in shaken flasks on a corn-steep-lactose medium supplemented with β-phenylethylamine. These cultures also produce only penicillin-G on this medium and thus differ from the parent culture (Wis. Q_{176}) in another respect, for the latter culture produces only 80% to 90% penicillin-G on this medium (7, 123, 254). If these cultures become widely used in the industry it will be a rather major change from the pigment-producing multi-penicillin-producing cultures used within the past few years.

PENICILLIN PRODUCTION BY CULTURES NOT BELONGING TO THE *Penicillium notatum-chrysogenum* GROUP. While attention has been concentrated on penicillin production by the *P. notatum-chrysogenum* group and cultures of this group seem to be characterized by penicillin production (192, 195, 243), many other *Penicillium* cultures have been reported to produce penicillin. Penicillin-like substances have been isolated from cultures of *P. avellaneum, P. rubens, P. turbatum* (73), *P. lanosum, P. roseo-citreum, P. griseo-fulvum* and *P. spinulosum* (195). It is interesting to note that cultures of *P. turbatum* and *P. spinulosum* have been found to produce penicillin and the antibiotic substance spinulosin (195).

Penicillin or penicillin-like substances have also been reported as metabolic products of a number of Aspergilli, including *Aspergillus flavus* (151, 152, 195, 242), *A. parasiticus* (4, 47), *A. nidulans* (61, 62, 82, 195), *A. giganteus* (184), *A. niger* (82), *A. flavipes* (251, 82, 195) and *A. oryzae* (82, 252). It has also been noted in culture filtrates from *Trichophyton mentagrophytes* (177) and a thermophilic fungus (205). Identification of penicillin as a metabolic product in these studies has usually been on the basis of antibiotic spectra and heat stability, and the antibiotic has rarely been isolated in pure form. Production by these cul-

tures apparently depends in part on the media used, as indicated in the studies with *A. parasiticus* (4, 47) where much higher antibiotic activity was noted when cornsteep water-containing media were used than when other media were employed.

While it is possible that further study of some of the above cultures will indicate that they may be more desirable than the strains of *P. chrysogenum* now in use, perhaps after selection procedures similar to those associated with the *P. chrysogenum* cultures, it is not too likely that the penicillin-producing industry will quickly transfer its operations to utilize these cultures. This reluctance to make such a change probably stems from the difficulty in revising recovery operations and other details associated with the production of penicillin which are only indirectly connected with the fermentation operations.

MEDIA USED FOR THE MYCOLOGICAL PRODUCTION OF PENICILLIN

HISTORICAL DEVELOPMENT. Successful production of large quantities of penicillin has depended in part on selection of cultures, as previously mentioned, and in part on the formulation of media suitable for the growth of these cultures. Fleming (70) grew his culture on a nutrient broth medium and noted that addition of glucose or sucrose to this medium apparently delayed production of the antibiotic principle. A modification of the Czapek-Dox medium (glucose and inorganic salts) was used by Clutterbuck et al. (43), and studies by the Oxford group (2) indicated that the sodium nitrate component of this medium could be replaced by peptone, the glucose by maltose or sucrose, and the proportions of the inorganic salts could be varied without marked effect on penicillin production. They also noted that addition of ten grams of yeast extract per liter to the medium resulted in a faster rate of production but apparently did not affect the quantities produced.

In other early reports (pre-1942) a number of other media were used, including veal broth (202), nutrient broth (20), tryptone-glucose-salts broth (69), amino acid mixture—brown sugar (69), a modified Czapek-Dox medium supplemented with peptone (150) and a modified Czapek-Dox medium in which brown sugar replaced glucose (150). In all of the studies (surface culture process) comparatively small quantities of penicillin were pro-

duced, usually only one to four units per ml. It was also generally observed that the penicillin-producing period was rather short, and if the pH of the fermentation was allowed to rise rapidly only small amounts of penicillin were found.

Perhaps the first outstanding improvement in the production of penicillin was associated with the observation made by Dr. A. J. Moyer in 1941 that addition of a small quantity of cornsteep liquor to the medium resulted in a manifold increase in penicillin production (44, 45, 159, 163). This material had been previously

TABLE V

GENERAL ANALYSIS OF CORNSTEEP LIQUOR

Constituent	grams/100 grams
Water	45–55
Total N (Kjeldahl)	2.7–4.5
Amino N (Van Slyke)	1.0–1.8
Volatile N	0.15–0.40
Free reducing sugar as glucose	0.1–11.0
Lactic acid	5–15
Volatile acids as acetic	0.1–0.3
SO_2	0.009–0.015
Ash	9–10
Some components of ash:	
Ca	0.25–0.75
Cu	0–0.0015
Fe	0.005–0.025
Mg	0.25–0.5
Mn	0.002–0.005
S	0.15
Zn	0.00025–0.0025
K	0.5–1

Abstracted from Liggett and Koffler (144)

used as a component of many fermentation media, a few of which are noted in the review by Liggett and Koffler (144), and was available in large quantities. During the period extending from 1942 to the present it has apparently been widely used as a major constituent of the media for production of penicillin and other antibiotic substances by microorganisms, and although other media have been proposed on which the mold will grow well and produce penicillin, none of these has apparently replaced the cornsteep liquor medium (88*a*). Further studies by Dr. Moyer and associates indicated that penicillin production on a medium containing

lactose as a carbohydrate source and cornsteep liquor as a nitrogen source was very high, and this medium has been used with some variations in most of the industrial production units (88*a*).

While this lactose-cornsteep liquor medium is relatively inexpensive and large quantities of the ingredients are available (44), a considerable amount of research has been expended in studying other media. Most of these media have contained a protein nitrogen source, a carbohydrate source, a mineral supplement and other ingredients, as will be mentioned in the following discussion. Several synthetic media in which the composition of all components is known have also been prepared, and, as will be indicated, penicillin production on a few of these has approached that on the cornsteep liquor–lactose medium, but none of these has been widely employed, usually for economic reasons.

NATURAL MEDIA. In discussing the various media which have been used for the culture of penicillin-producing fungi, it is desirable to divide them into two groups, the natural media and the synthetic media. At least one constituent of the former is a natural product whose exact composition is unknown, for example, yeast extract, cornsteep liquor, cottonseed oil meal. A synthetic medium is one whose composition is known, and, although it may contain ingredients of natural origin, these are highly purified and there is little chance that substances of unknown composition have been introduced. As in most media for the growth of fungi, the media used in producing penicillin have contained a nitrogen source, a carbohydrate source or energy source, and a number of minerals which are required for the growth of the fungus and indirectly perhaps for the production of penicillin. In the natural media protein nitrogen sources have been added rather than inorganic nitrogen, while in the synthetic media both organic and inorganic nitrogen-containing compounds have furnished the nitrogen required.

PROTEIN NITROGEN SOURCE. As has been mentioned above, the work of the Oxford group (2) indicated that addition of yeast extract at a level of 100 grams per liter to a basal medium containing per liter: sodium nitrate, three grams; monobasic potassium phosphate, one gram; potassium chloride, 0.5 gram; magnesium sulfate heptahydrate, 0.5 gram; ferrous sulfate heptahydrate, 0.01 gram; glucose, 40 grams; and tap or distilled water

to one liter resulted in an increase in the rate of penicillin production but not in an increase in the quantity of this antibiotic. When the Fleming culture was grown on this medium (surface culture method) potencies of one to four units per ml. were obtained after a fermentation period of six to nine days. Addition of other supplements to this medium did not result in increased penicillin yields. Studies in other laboratories suggested that such materials as Amigen, a commercially available digest of purified casein and pork pancreas in which the proteins have hydrolyzed by enzmatic action, would serve as nitrogen source (233), but higher yields of penicillin were not obtained.

As has been mentioned, Dr. A. J. Moyer observed that cultures grown on the medium used by Abraham et al. (2) supplemented with two to five ml. of cornsteep liquor produced ten units per ml. of penicillin, and that increasing the concentration of cornsteep liquor in the medium to 100 to 125 grams per liter resulted in yields of 60 to 80 units (surface culture process) after other minor changes in the medium (159). Further experimentation indicated that the following medium perhaps contained the optimal concentration of ingredients for penicillin production of culture NRRL 1249.B21 (grams per liter): sodium nitrate, 3; magnesium sulfate heptahydrate, 0.5; monobasic potassium phosphate, 1; glucose monohydrate, 2.75; zinc sulfate heptahydrate, 0.044; manganese sulfate quadrahydrate, 0.004; lactose monohydrate, 44; cornsteep liquor, 100; and water to one liter. This medium resembles in part those used by earlier investigators and has been the basis for other studies in many laboratories. In some experiments yields approaching 200 units per ml. were obtained (159). The inclusion of cornsteep liquor is no doubt responsible for the increase in penicillin production, and perhaps a brief description of its manufacture, abstracted in part from Liggett and Koffler (144), is in order:

"Since cornsteep liquor is a by-product of the wet-milling industry it would be insufficient to discuss its manufacture apart from the whole process in which corn, after having been shelled and air-cleaned, is soaked, and then fractionated into its principal components by a combination of flotation and wet-screening procedures".

"The corn is first soaked, or steeped in open wooden tanks at 45–50° C for 40 to 48 hours. Five to seven gallons of water are required for every bushel of corn. The water used in steeping is process water that has been

used previously in other phases of the process, for example, the overflow from the gluten settling tank. During steeping the soluble materials are dissolved, the corn is softened, and its structure weakened and broken, which facilitates the grinding and further separation of its components. Just before the process water enters the tanks, SO_2 is added to prevent putrefaction and to assist in the extraction of the soluble compounds. The concentration of SO_2 is initially from 0.1 to 0.2%, but since most of the SO_2 is absorbed by the corn, it is lowered to 0.05% five hours after addition and to 0.01% within two hours. Moving in a general counter-current fashion, the most dilute water is placed on corn that has been steeped the longest and is transferred continuously in the direction of the corn most recently introduced. In this manner, the steep water having the highest concentration of solutes is used on corn just entering the system after which procedure the water is withdrawn and concentrated to a solid content of approximately 50%. This concentrated crude cornsteep liquor may then either be combined with gluten and fibrous materials and sold as animal feed, or used for microbiological purposes with or without further processing".

"The main disadvantage of cornsteep liquor in microbiology is its variable composition. This variability may depend somewhat upon the type and condition of corn, but even more upon a multitude of variables involved in the processing of starch. On the other hand, cornsteep liquor is an inexpensive alternative (4 to 6 cents per pound of liquor) to much more expensive materials such as yeast extract and peptone".

"Cornsteep liquor has a pH of 3.7 to 4.1, a specific gravity of 1.25, on proximate analysis a general composition as indicated in Table V. On the average 6.9% of the corn solids and 30% of the corn nitrogen are found in the steep liquor. Approximately 95% of the total nitrogen in cornsteep liquor is accounted for, after hydrolysis, by ammonia and the following amino acids: alanine, arginine, aspartic acid, cystine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tyrosine, and valine, with more than one-quarter of the nitrogen present as alanine (34). It also contains considerable amounts of the B-complex vitamins with the exception of thiamine (232). The ash of cornsteep liquor contains a wealth of mineral mineral elements, especially calcium, iron, magnesium, phosphorus, and potassium".

While the penicillin industry has used large quantities of cornsteep liquor during the past few years, the demand for this purpose has not exceeded more than 15% of that produced, with major production going to the live-stock feed industry. Under these conditions its has been rather difficult for the penicillin producers to arrange for supplies of this material which are of reasonable constant composition, and some variations in penicillin production have been attributed to poor cornsteep liquor (45). This was especially noticeable in 1945 when a considerable drop

in the total production of penicillin in the United States occurred during the early fall months when the cornsteep liquor made with the poorer grade corn was being used in the preparation of media. Some effort has been made to produce a product which is standardized and perhaps better suited for inclusion in media used for the production of penicillin by alkali, fermentation and/or heat treatments (165, 169, 234). Some improvement in penicillin production of media prepared with poor cornsteep liquor is claimed by treatment of the medium with activated carbon (33).

The increase in penicillin yields noted after addition of cornsteep liquor to the culture medium was so marked that some effort was expended in investigating the specific role of the liquor in the metabolism of the mold and the production of penicillin. The liquor, as can be seen from Table V, supplies large quantities of nitrogenous materials, minerals (including the trace elements) and, as will be mentioned later, compounds which act as penicillin precursors. Addition of minerals and growth factors to media containing cornsteep liquor did not result in increased penicillin production in surface culture (159). Fractionation of the liquor by a number of procedures did not indicate which factors were responsible for the promotion of penicillin production (159), although some stimulation was obtained when certain amino acids present in cornsteep liquor were added to synthetic media (31*a*, 99*a*, 252). Later investigations indicated that the liquor contains appreciable quantities of phenethyl and phenacetyl derivatives which probably act as precursors for the production of penicillin, but this was not recognized until after considerable work had been done on the chemistry of the penicillins, and perhaps should be considered a by-product of these latter studies. As has been mentioned earlier, increasing the concentration of cornsteep liquor in media used for surface culture resulted in increase in penicillin production, and, as indicated in Table VI, this same effect was observed when submerged culture methods were used. Addition of a buffering agent is apparently desirable in the submerged culture method (84, 160, 161), while it may not be necessary in the surface culture process (159), and, as indicated in Table VI, calcium carbonate has been found suitable for this purpose.

A large number of naturally occurring products have been

tested for penicillin-promoting activity similar to that noted when cornsteep liquor was added to the medium. The results obtained in various laboratories have varied somewhat with the cultures and processes used, but in general indicate that addition of such materials as hydrolyzed casein, hydrolyzed corn, wheat or soybeans to a basal medium containing inorganic salts and carbohydrate did not stimulate penicillin production to the extent noted when cornsteep liquor was added (79, 84, 159, 160, 162, 163, 164, 93). This once was interpreted as an indication that some factor present in cornsteep liquor is essential for the production of peni-

TABLE VI

EFFECT OF NUTRIENT LEVEL ON PENICILLIN PRODUCTION

Composition of medium				Maximum penicillin yield	Age at maximum yield
Cornsteep liquor (solids)	Lactose	$CaCO_3$	Salts*		
g/100 ml	g/100 ml	g/100 ml		units/ml	hours
2	2	0.2	+	237	54
4	3	1	-	480	64
6	3	1	-	636	67

*Salt mixture (grams/l): KH_2PO_4, 0.25 g; $MgSO_4 \cdot 7H_2O$, 0.12 g; $NaNO_3$, 1.5 g; at low cornsteep liquor concentrations the use of an auxiliary nitrogen source is desirable.

Fermentations conducted in 80 gallon tanks containing 220 l. of medium with aeration at rate of 200 liters per minute, and agitation by 12 in. propellors revolving at 270 r.p.m. and temperature control at 23° C. *Penicillium chrysogenum* X-1612.

Abstracted from Johnson et al.(121).

cillin (22), but later studies with synthetic media and with media prepared with seed meals indicate that cornsteep liquor is not exceptional in its penicillin-promoting ability (85, 87, 79, 179, 93). Indeed, as indicated in Table VII, penicillin production may at times be higher on media prepared with certain seed meals than with media prepared with cornsteep liquor. Less promising results have been obtained with chestnut extract (173) and pea extracts (47, 48, 49, 50, 51), and, while peptone has been found suitable in one instance (236) it has not been useful in others (159, 179) when compared with cornsteep liquor. Some of these

materials, notably the seed meals, are available in large quantities and are perhaps of less variable composition than the cornsteep liquor and may find use as ingredients of media for the production

TABLE VII

PRODUCTION OF PENICILLIN ON NATURAL MEDIA

Substance substituted for cornsteep liquor*		Maximum penicillin without precursor	Production with precursor
	g/l	U/ml	U/ml
Cornsteep liquor (solids)	20	650	1,300
	30	580	1,050
	40	400	800
Cottonseed oil meal	30	800	1,200
	40	1,000	1,160
Linseed oil meal	20	700	1,035
	40	635	1,100
	60	...	570
Coconut oil meal	60	450	1,150
	90	500	810
	120	300	680
Sardine meal	10	150	610
	20	400	900
	30	575	1,130
Rape seed oil meal	30	285	515
	60	730	705
	90	335	615
Peanut oil meal	20	600	630
	30	950	900
	40	1,050	1,350
Mustard flour	30	650	980
	40	590	1,060
Soybean meal (expeller).	20	305	1,030
	40	170	930
Castor bean meal	20	225	940
	40	300	580
Fish meal (mixed)	20	830	430
	40	540	790
Menhaden meal	20	780	390
	40	460	430

*Other ingredients included 30 g. lactose and 1 g. phenylacetylethanolamine as penicillin G precursor (where indicated), 3.2 ml. soybean oil, and 10 g. calcium carbonate per liter.

Adapted from Perlman (179).

of penicillin in certain sections of the world where cornsteep liquor is not available.

Carbohydrate Constituents and Energy Sources. When the Czapek-Dox medium (2) or slight modifications of it (43, 59, 79, 150) were used in the surface culture process there was no marked difference in response to various carbon sources, as indicated by penicillin production, except that lactose was inferior because it supported only a trace of fungus growth (159). The slight increase resulting by substitution of brown sugar (86, 150) for the glucose could be attributed to the presence of metallic impurities in the brown sugar. However, once the effectiveness of cornsteep liquor was recognized and this material was added to the basic medium, a marked effect of various carbon sources on penicillin yield was observed (159). There was sufficient assimilable carbohydrate, probably mainly glucose and dextrins, to support good fungus growth and moderate penicillin production, even in the absence of added carbon sources. Lactose, corn starch, and corn dextrin were equally good for penicillin production (159, 176, 168) with glucose, glycerol, sorbitol and brown sugar (sucrose) being significantly inferior. Crude starch preparations, including ground corn, ground wheat and granular wheat flour, could be used in this surface culture process (159). In one report addition of corn oil to a modified Czapek-Dox medium containing brown sugar as a glucose replacement resulted in a significant increase in penicillin production, while addition of lactose to this medium did not (112).

In certain laboratories comparable studies on carbohydrate sources in submerged cultures suggested that higher penicillin potencies were obtainable when a cornsteep–brown sugar medium was used instead of a cornsteep-lactose or a cornsteep-glucose medium (84, 86). Sucrose and glycerol could be substituted for brown sugar without appreciable change in penicillin production, but replacement of brown sugar with cane molasses and other crude sucrose sources was not always satisfactory. In other laboratories higher yields were obtained with a cornsteep-lactose medium (44, 160, 164), and apparently this medium served in some manufacturing plants, while the cornsteep liquor–brown sugar medium was used in others. This apparent discrepancy between results in different laboratories may be partially explained

by the studies on the effect of aeration on penicillin production when the fungus was grown on these different media (135, 182), and is probable that there were considerable differences in aeration efficiency in the various penicillin-manufacturing plants. Among the carbohydrates that may be substituted for lactose are sorbitol, mannitol and *a*-methyl glucoside, while starch, inulin, fructose, glucose, galactose, sorbose and xylose do not appear to serve as satisfactory substitutes (178, 179). Crude lactose sources, including cheese whey, may be used as replacements for the purified product (179) without appreciable effect on penicillin yield, while

TABLE VIII

EFFECT OF ADDITION OF PHENYLACETYL DERIVATIVES ON THE PRODUCTION OF PENICILLIN*

Phenylacetyl derivatives added**	Units of penicillin per ml	Penicillin G content of penicillins produced
None	242	0
Phenylacetic acid	490	82
Phenylacetamide	504	71
Phenylacetyl-*dl*-alanine	483	57
Phenylacetyl glycine	461	56
β-phenylethylamine	428	51
dl-phenylalanine	220	24

*Abstracted from Singh and Johnson (218).

**Quantity of derivative equivalent to 0.14% phenylacetic acid was added to cultures of *Penicillium chrysogenum* Wisconsin Q_{176} (growing on synthetic medium) 24 hours after inoculation with a vegetative inoculum.

casein whey apparently cannot be employed for this purpose (179). Lactose is metabolized slowly under these aeration conditions (135, 178) and requires less oxygen for utilization, while simpler sugars are metabolized rapidly and require higher aeration rates. Mixtures of lactose and glucose or lactose and sucrose apparently may be substituted for lactose without appreciable effect on penicillin production (178). The fungus produces amylases and invertase which hydrolyze the polysaccharide and sucrose quickly (85, 178) but apparently does not produce lactase or an enzyme splitting *a*-methyl glucoside (178).

INORGANIC CONSTITUENTS. The ash content of cornsteep liquor supplies a considerable quantity of minerals to the medium, and supplementation of media containing this material with trace elements and other salts has not resulted in considerable change in penicillin production (159). As has been mentioned before, Dr. Moyer added traces of zinc sulfate and manganese sulfate to his media, but no definite need could be established for these minerals when the basal medium contained cornsteep liquor (144). Addition of iron salts to the cornsteep liquor medium, either as an ingredient of the medium or by erosion from iron equipment, resulted in decrease in penicillin yields when more than 30 p. p. m. were present (226). Addition of sodium nitrate, magnesium sulfate and potassium phosphate (85, 159, 160) did result in increase in penicillin production, and several studies have been made to determine the optimum concentrations of these ingredients for use in the surface culture (186, 187) and the submerged culture process (188). However, as penicillin production was not greatly decreased when these materials were eliminated from cornsteep water–lactose or seed meal–lactose (22, 179) media, it is probable that they are not of major importance, although it may be desirable to add them to supplement those quantities of nitrogen, sulfur and phosphorus already in the medium from the cornsteep liquor. Addition of borax to a cornsteep water–lactose medium appeared to stimulate penicillin production by several cultures of *P. chrysogenum* but had little effect on others (127, 136, 137*a*).

Addition of calcium carbonate to cornsteep liquor–lactose media in the submerged culture process has already been mentioned. Substitution of other alkali metal carbonates in these media for calcium carbonate did not result in as high penicillin yields as with calcium carbonate (178), and use of sodium or potassium hydroxide to control the pH of the media resulted in decreased penicillin production (121, 226). While this may suggest a calcium requirement for the mold, the high yields obtained when synthetic media, which contain only trace amounts of calcium salts, are used, do not substantiate this hypothesis.

PENICILLIN PRECURSORS. As the chemistry of the penicillins was examined during the early days of production development, it soon became evident that more than one compound exists which

has the pharmacological properties associated with the name "penicillin". American workers isolated a pure crystalline penicillin termed "penicillin-G" (41, 255), which was characterized by having phenylacetic acid as a degradation product, whereas the penicillin studied by the English workers, termed "penicillin-F", contained the Δ-2-pentenyl radical, as indicated in Table I, and did not yield phenylacetic acid as a degradation product. Moyer and Coghill (46, 161), in studying the production of penicillin in surface and submerged culture, added a number of degradation products of penicillin to the culture medium. When phenylacetic acid was added an increase in the total quantity of penicillins produced was noted, and the proportion of penicillin-G also seemed to increase, as measured by bioassay and isolation procedures. In these studies the quantity of phenylacetic acid converted to penicillin-G was less than 2% of that added to the fermentation, but this observation was of great practical importance, for it permitted direction of the fermentation to the production of the desired penicillin-G, and it is probable that all media now used commercially contain added penicillin-G precursor.

Studies in other laboratories (9, 41) utilizing phenylacetic acid marked with deuterium established that some of the phenylacetic acid added to fermentations was converted eventually to penicillin-G. Later experiments (55) have indicated that if phenylacetamide labeled with C^{13} at the amide group is added to a growing culture, the penicillin-G isolated from this culture will contain the isotopic tracer in the expected position. These labeled penicillins have been used in analysis of penicillins for penicillin-G by the isotopic dilution method.

A number of derivatives of phenylacetic acid and related compounds have served in these studies, including phenylethylamine, phenylacetamide, phenaceturic acid, phenylacetylethanol amine and derivatives of phenylacetamide (41, 85, 87, 88, 95, 103, 142, 143, 155, 186, 190, 218, 237). The efficiency of the conversion of some of these compounds to penicillin-G as calculated from the data presented in Table VIII, is less than 5%. This varies somewhat from one compound to another and also probably depends in part on the strain of *P. chrysogenum* and the cultural conditions (260). Addition of phenylacetylethanol amine to the culture medium has

also been shown to increase the production of an unidentified antibiotic, presumably penicillin-G, by cultures of the *Aspergillus nidulans* group (62).

This observation that addition of phenylacetic acid to growing cultures resulted in formation of penicillin-G prompted Behrens and associates (9–15) to add other compounds to the medium, including substituted phenacetyl derivatives, containing halogens and other groups, and other organic radicals. They were successful in isolating a number of penicillins in crystalline form which contained the added material in the R-position (see Table I) and which apparently did not occur naturally when the fungus was grown on the usual media. These are often termed "artificial penicillins" to distinguish them from those called "natural penicillins," produced when the fungus is grown on synthetic or cornsteep-liquor-containing media. Obviously the distinction is rather arbitrary. Recent experiments (25) have indicated that addition of some of the *n*-fatty acids or glycerides of these fatty acids to synthetic or cornsteep-water-containing media resulted in production of the corresponding penicillin containing an aliphatic grouping in the R-position.

The difference in the types of penicillins produced in the United States and England was eventually explained by the observation that cornsteep water which was widely used in the former country contains appreciable quantities of phenethylamine and phenylalanine (41, 88, 154). These compounds apparently act as penicillin-G precursors and account for the penicillin produced when cultures are grown on cornsteep-water-containing media. It is probable that the phenylalanine content of the protein constituents of the seed meal media (85, 87, 179) acts in the same manner. Present indications are that practically no penicillin-G is produced when cultures of *P. chrysogenum* are grown on synthetic media which do not contain added phenylacetyl derivatives (123), although the mold mycelium contains approximately 2% phenylalanine (182). If this is found to be generally true, penicillin-G should be really classified as an artificial penicillin and probably would have never been found if Dr. Moyer had not added cornsteep water to his media.

Cornsteep water was also found to have some tyramine, presumably derived by bacterial decarboxylation of tyrosine, which

was assumed to serve as a precursor for the production of penicillin-X (88, 154). Addition of tyrosine or tyramine to growing cultures resulted in the production of penicillin-X (5, 35, 95, 142, 143, 155). As has been mentioned previously, certain cultures appear to be predisposed to the production of one penicillin over another, and perhaps the most notable is the group producing large quantities of chloroform-insoluble penicillin-X (194, 196–198).

While a number of compounds have been studied as possible precursors for the other portions of the penicillin molecule, no reports have demonstrated that any compounds have such activity when added to growing cultures (9, 229, 230), although addition of a number of amino acids has resulted in some increase in penicillin production which may be through this mechanism (229, 231).

SYNTHETIC MEDIA. Concurrent with the studies on the production of penicillin by fungi grown on natural media, as mentioned above, investigations were in progress studying penicillin production by these fungi when grown on synthetic media. A large number of media formulations were tested with varying results. The composition of several of the more successful from the point of view of penicillin production are listed in Table IX with some indication of the antibiotic yields obtained. The highest yields of penicillin were achieved on media which resembled in part the cornsteep liquor–lactose media in that they contained those ingredients or similar materials found in the cornsteep water. Although the yields on these synthetic media approach those obtained on the cornsteep-liquor media, no reports are available which suggest that they have been used in larger than pilot-plant units.

While most cultures of *Penicillium chrysogenum* will grow rather well on a number of synthetic media, penicillin yields are not always associated with good growth, as measured by quantity of mycelium formed. Jarvis and Johnson (114–116) have evaluated the role that certain constituents of a synthetic medium play in the production of penicillin. Their observations led them to conclude that the rate of formation of penicillin appeared to reach a maximum under conditions which supported only a very slow growth rate. As indicated in Table IX, they obtained yields of over 300 units per ml. on a medium which contained lactose, glucose, ammonia, acetate, lactate and inorganic salts, and after supplementation with a penicillin-G precursor, yields increased to

TABLE IX

PENICILLIN PRODUCTION ON SYNTHETIC MEDIA

A. Surface Culture Process

Composition of medium:		
lactose, 30 g	lactose, 30 g	lactose, 15 g
glucose, 10 g	glucose, 10 g	glucose, 15 g
citric acid, 15 g	starch, 15 g	$NaNO_3$, 6 g
acetic acid, 2.5 g	acetic acid, 2.5 g	KCl, 0.5 g
phenylacetic acid, 0.5 g	citric acid, 10 g	$MgSO_4 \cdot 7H_2O$, 0.5 g
$MgCl_2 \cdot 6H_2O$, 0.5 g	phenylacetic acid, 0.5 g	Succinic acid, 10 g
$FeCl_3$, 0.05 g	$(NH_4)_2SO_4$, 5 g	Glutamic acid, 4 g
$Cu(NO_3)_2 \cdot 3H_2O$, 0.02 g	Ethylamine, 3 g	Arginine, 0.3 g
$ZnCl_2$, 0.02 g	$MgSO_4 \cdot 7H_2O$, 0.5 g	Histidine, 0.3 g
$MnCl_2 \cdot 4H_2O$, 0.02 g	KH_2PO_4, 1 g	KH_2PO_4, 1.5 g
$Co(NO_3)_2 \cdot 6H_2O$, 0.02 g	$CuSO_4 \cdot 5H_2O$, 0.01 g	$ZnSO_4 \cdot 7H_2O$, 0.001 g
Na_2SO_4, 1 g	$FeSO_4 \cdot 7H_2O$, 0.02 g	$FeSO_4 \cdot 6H_2O$, 0.010 g
NH_4NO_3, 5 g	$ZnSO_4 \cdot 7H_2O$, 0.01 g	$MnSO_4 \cdot 5H_2O$, 0.01 g
NaF, 0.01 g	$MnSO_4 \cdot 4H_2O$, 0.01 g	$CuSO_4 \cdot 5H_2O$, 0.005 g
Distilled water to 1 liter (pH adjusted to 5.8—with KOH)	$Co(NO_3)_2 \cdot 6H_2O$, 0.02 g	Distilled water to 1 liter (pH adjusted to 5.7–5.8)
	NaF, 0.01 g	
	Distilled water to 1 liter (pH adjusted to 5.6–5.9 with KOH)	
Inoculum:		
Spore suspension	Spore suspension	Spore suspension
Volume of fermentation:		
100 ml	100 ml	100 ml
Culture used:		
NRRL 1249.B21	NRRL 1249.B21	NRRL 1249.B21
Maximum yield:		
70 Units/ml	119 Units/ml	205 Units/ml
Reference:		
107	31a	252

B. Submerged Culture Process

Composition of medium:		
lactose, (monohydrate), 22.5 g	lactose, 30 g	lactose, 15 g
glucose, 7.5 g	glucose, 10 g	glucose, 5 g
ammonium acetate, 3 g	ammonium acetate, 3.5 g	acetic acid, 4 g
ammonium lactate, 5 g	ammonium lactate, 5.5 g	NH_4NO_3, 5 g
KH_2PO_4, 3 g	KH_2PO_4, 3 g	KNO_3, 3.5 g
$MgSO_4 \cdot 7H_2O$, 0.25 g	$MgSO_4 \cdot 7H_2O$, 0.25 g	KH_2PO_4, 2 g
$FeSO_4 \cdot 7H_2O$, 0.1 g	$FeSO_4 \cdot 7H_2O$, 0.1 g	$MgSO_4 \cdot 7H_2O$, 0.5 g
$CuSO_4 \cdot 5H_2O$, 0.005 g	$CuSO_4 \cdot 5H_2O$, 0.005 g	$FeSO_4 \cdot 7H_2O$, 0.2 g
$ZnSO_4 \cdot 7H_2O$, 0.02 g	$ZnSO_4 \cdot 7H_2O$, 0.02 g	$ZnSO_4 \cdot 7H_2O$, 0.04 g
Na_2SO_4, 0.5 g	Na_2SO_4, 0.5 g	$CuSO_4 \cdot 5H_2O$, 0.005 g
$MnSO_4 \cdot 4H_2O$, 0.02 g	$MnSO_4 \cdot 4H_2O$, 0.02 g	phenylacetamide, 0.25 g
$CaCl_2 \cdot 2H_2O$, 0.05 g	$CaCl_2 \cdot 2H_2O$, 0.05 g	distilled water to 1 liter
distilled water to 1 liter	phenylacetic acid (in 9 additions during fermentation), 14.5 g	pH adjusted to 6 with KOH
	distilled water to 1 liter	

TABLE IX (continued)

Inoculum: 3% of vegetative growth grown on glucose-salts medium	1.5% of vegetative growth grown on glucose-salts medium	10^3 germinated spores grown on cornsteep water medium
Size of fermentation: 80 ml in 500 ml Erlenmeyer flask	80 ml in 500 ml Erlenmeyer flask	50 gallons in glass-lined tank
Culture: *P. chrysogenum* Wis. Q_{176}	*P. chrysogenum* Wis. Q_{176}	*P. chrysogenum* Wis. Q_{176a}
Maximum penicillin yield: 360 U/ml	915 U/ml	245 U/ml
Reference: 116	218	97

over 500 units per ml. (116, 218). Analyses of the penicillin types present in media in which these precursors were not added indicated that little penicillin-G was produced in the absence of a phenylacetyl derivative (103, 104, 116, 123, 218). This suggests that the major portion of the penicillins produced on other synthetic media (2, 41*a*, 75*a*, 79, 99*a*, 126*a*, 137*a*, 137, 189, 190, 231, 252) was probably of the aliphatic type. Such a conclusion is difficult to substantiate, as the analytical methods used during the period 1943–1946 were not very specific with regard to determination of certain types of penicillins.

A rather large number of observations were made on the effects of certain inorganic elements on the production of penicillin by cultures grown in both surface and submerged culture processes (41*a*, 85, 103, 116, 126*a*, 137, 137*a*, 127, 190, 178). Addition of the ash obtained from cornsteep water (126*a*, 127) or from cottonseed oil meal (84) to synthetic media resulted in increased penicillin production. The activity of the former preparation was found to be associated with its iron and phosphate content (137*a*). A requirement for other elements, e. g., chromium, has been noted in one laboratory (189, 190) but has not been confirmed elsewhere (116, 137*a*). A number of sulfur compounds have been tested (35, 107, 142, 149) and, while poor growth was noted on a sulfur-free medium, inorganic sulfate was as effective as any compound in stimulation of growth and penicillin production.

Quantitative studies have been made (116*a*) on the sulfur, potassium, magnesium, phosphorus and iron requirements of *Penicillium*

chrysogenum Q_{176} for the production of penicillin when grown on a synthetic medium. The iron, phosphorus and sulfur requirements for normal penicillin production were, respectively, 20 times, two times, and about one and one-half times those for normal growth. For potassium and magnesium, normal penicillin production was obtained at the same metal level as that required for normal growth.

Supplementation of synthetic basal media with some of the amino acids found in cornsteep water has resulted in some increase in penicillin production under certain conditions (31*a*, 51, 99, 229, 231, 252, 256, 257). Study of these reports suggests that perhaps the basal media used in these experiments were deficient in some essential nutrient, although it is possible that some of these substances are more easily assimilated as carbon and nitrogen sources than as ammonium lactate or ammonium acetate.

STUDIES ON THE BIOSYNTHESIS OF THE PENICILLINS

Studies on the metabolism of the penicillin-producing fungi were started shortly after the development of media which supported growth and penicillin production. These studies included observations on the chemical changes brought about on the medium constituents by the growth of the mold, study of some of the enzyme systems of the penicillin-producing fungi, investigation of the effects of various physical conditions on the metabolism of the fungus and penicillin production, testing of certain compounds as possible precursors or intermediates in the biosynthesis of the penicillins, and preliminary study of biosynthesis of possible intermediates in the biosynthesis of the penicillins.

As will be indicated below, a considerable amount of time has been spent studying the chemical changes occurring during the fermentation of various types of media, trying to correlate these changes with penicillin production. Unfortunately the information collected has not indicated at just what stage in the metabolism of the mold penicillin is formed, and actually little more is known of the mechanism of penicillin formation than was known ten years ago. However, these studies have served to explain in part why penicillin production on certain media is high or low under certain physical conditions, and this information has been of some value in the manufacture of penicillin. After surveying

the reports which have appeared concerning this topic, it is apparent that much remains to be done before satisfactory explanations are available to explain why some cultures are good penicillin producers and others are poor ones.

CHEMICAL CHANGES OCCURRING DURING FERMENTATION OF VARIOUS MEDIA BY PENICILLIN-PRODUCING FUNGI. The many studies on the chemical changes occurring during the fermentation of various media by penicillin-producing fungi when grown in

TABLE X

CHANGES CHARACTERIZING THE THREE PHASES OF PENICILLIN FORMATION (LACTOSE-CORNSTEEP WATER MEDIUM)

	Phase 1	Phase 2	Phase 3
Penicillin	Slight production	Maximum rate of production	Concentration falls
pH	Sharp rise	Plateau or slight drop	Rise
Mycelium	Rapid growth, high N content	Slow growth, N content lower	Decrease in weight and N content
Lactose	Used slowly	Used more rapidly	Small remaining amount exhausted
Lactic acid	Exhausted rapidly		
Ammonia	Released into medium	Utilized	Released into medium
Nitrate	Used at maximum rate, although slowly	Slow use	Slow use, not exhausted
Nonammonia Kjeldahl N	Used extensively	Concentration rather stable	Concentration increases
Inorganic phosphorus	Used at maximum rate, although slowly	Slow use	No use or liberation
Q_{O_2} (N)	Maximum	Decreases	Minimum

Abstracted from Koffler et al. (135).

surface culture (31*a*, 79, 107, 108) and submerged culture (84, 92, 88*a*, 97, 114–116, 120, 135, 137, 138, 160, 179, 218, 226, 227) have indicated in part the conditions which are most favorable for penicillin production without apparently indicating what parts of the metabolic systems of the mold are actually involved in the production of penicillin. The role of certain constituents of synthetic (114–116, 127, 128, 136, 137*a*) and natural media (22, 179, 186, 187, 188) have been evaluated but have not shed much light on this problem. Usually the submerged culture fermentations may

be divided into three phases, as indicated in Table X, on the basis of the metabolic changes noted, and combining these observations with measurements of the penicillin yields at various stages has permitted speculation on the optimum conditions for penicillin production (115, 120, 227). Sometimes the conditions postulated have been reproduced with high yields of penicillin, and

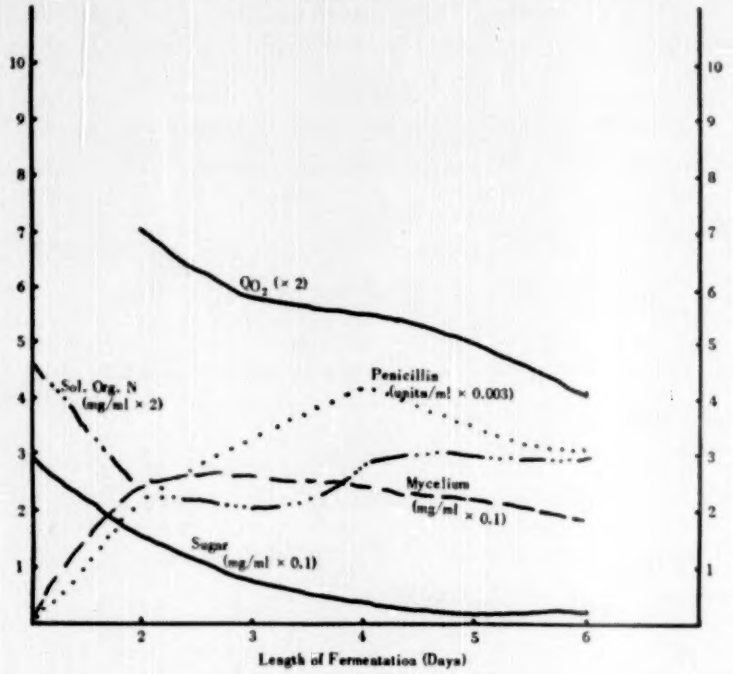


FIG. 3A. Chemical changes occurring during fermentation of cornsteep water medium by *Penicillium chrysogenum* Wisconsin Q_{176} (Shaken Flask). Medium composition (grams per liter) : cornsteep water solids, 20; lactose, 30; $CaCO_3$, 10; N(2-hydroxyethyl)phenylacetamide, 1; soybean oil, 2.8; water to 1 liter. (Abstracted from Perlman (180)).

unfortunately for these theories, at other times when these conditions have been met, penicillin yields have been low.

The changes occurring during fermentation of a cornsteep water–lactose or a cornsteep water–sucrose medium by non-penicillin-producing cultures were found to closely resemble those occurring during fermentation of these media by penicillin-producing

cultures (135), suggesting that the changes noted were not important as far as penicillin production was concerned. Also, as indicated in Figs. 3A and 3B, the changes occurring when relatively poor penicillin-producing cultures are used are nearly the same as when good penicillin-producing cultures are studied, and it has apparently not been possible to select new cultures on any

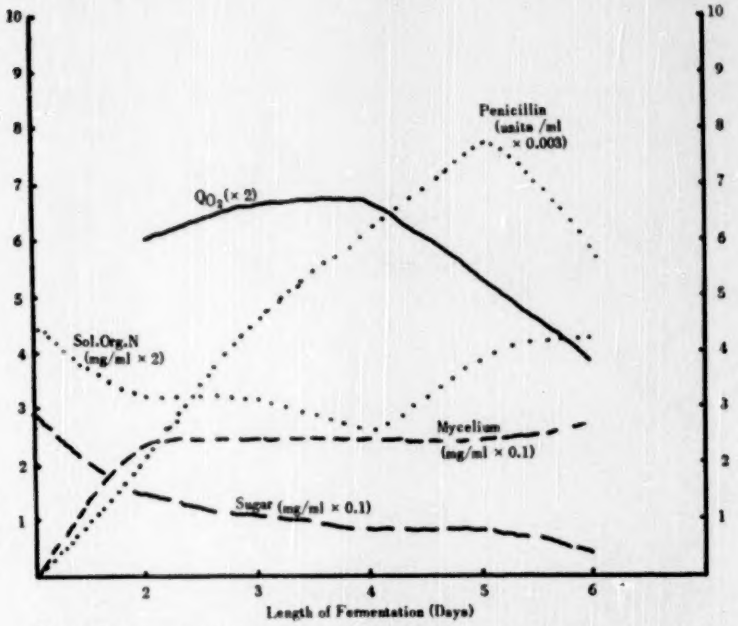


FIG. 3B. Chemical changes occurring during fermentation of cornsteep water medium by variant of *Penicillium chrysogenum* Wisconsin Q_{176} (Shaken Flask).

Medium composition (grams per liter): cornsteep water solids, 20; lactose, 30; $CaCO_3$, 10; N(2-hydroxyethyl)phenylacetamide, 1; soybean oil, 3.2 ml.; water to 1 liter. (Abstracted from Perlman (180)).

other basis than penicillin production. This situation is further complicated by the observations that certain cultures produce large amounts of one type of penicillin from a given medium, while others produce large amounts of another type from the same medium (7, 25–27, 194).

In general the sequence of chemical changes noted in small fermentations conducted in shaken flasks (135, 179) has been

observed in studies in large units (26–28, 120, 226). Some of the data collected in stirred jar fermenters are presented in Figs. 4 and 5. Alterations in the temperature of the fermentations (178, 226, 227), the aeration rates (26, 28, 97, 23), the methods of

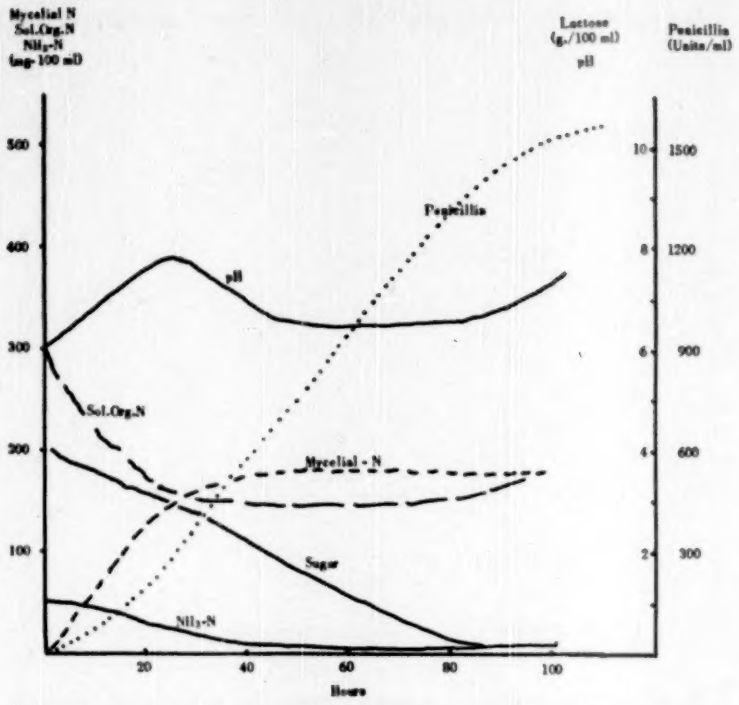


FIG. 4. Chemical changes occurring in stirred jar fermentations producing highest yields of penicillin from *Penicillium chrysogenum* Wisconsin Q_{176}. Medium composition (grams per liter): cornsteep water solids, 40; lactose, 2.5; glucose, 10; $CaCO_3$, 10; Na_2SO_4, 1; ammonium acetate, 1.5; water to 1 liter. (Abstracted from Peterson (82)).

agitation and aeration (26, 27, 97, 226), and the medium composition (26, 28, 115, 116, 97) have some effect on the duration of some of these phases but have not been found to affect the sequence of these changes markedly (97, 115, 116, 135, 178). Such alterations as pH control by addition of acid or alkali (28, 226, 227) and continuous addition of ingredients (28, 160, 163) have at times

prolonged the penicillin production phase slightly, but the results obtained in these experiments have not been so useful as had been expected. Study in flask and stirred jar fermentation units of the oxygen demand when various carbohydrates have been present in the media as energy sources has indicated (120, 135, 179, 256, 257) that higher aeration rates are required to obtain high peni-

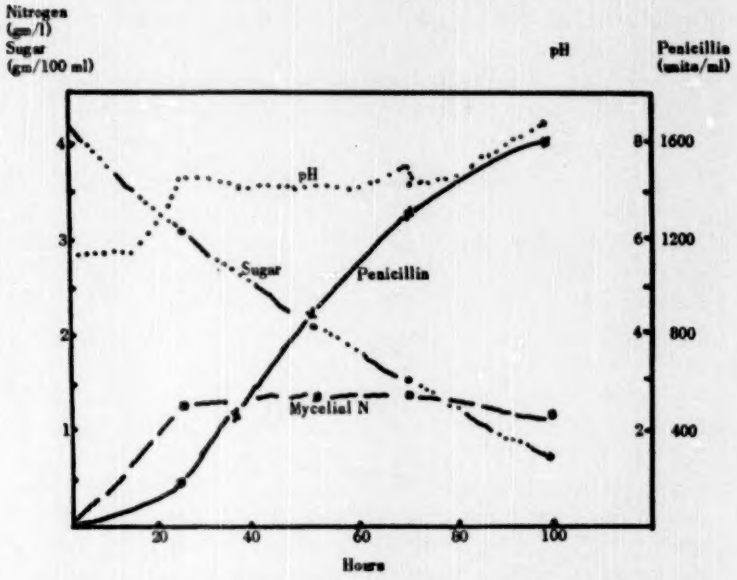


FIG. 5. Chemical changes occurring in stirred jar fermentation on *Penicillium chrysogenum* Wisconsin Q_{176} with relatively stable pH control.
Medium composition (grams per liter): cornsteep water solids, 40; lactose, 25; glucose, 9.1; Na_2SO_4, 1; $CaCO_3$, 5; phenylacetic acid, 3.5, added in 12 portions at 12-hour intervals; water to 1 liter. (Abstracted from Brown and Peterson (28)).

cillin yields when the media contain glucose, fructose, sucrose, starch and dextrin, singly or in combination, than when lactose, mannitol or α-methyl glucoside, which are more slowly oxidized by the fungus, are used (135, 178, 179, 257).

Most of the above studies were made on cultures grown on media containing cornsteep water as a nitrogen and partial carbon source. When linseed oil meal or cottonseed oil meal were substituted for the cornsteep water, approximately the same changes

were noted (179) as had been found with the cornsteep-water-containing media. Formulation of several synthetic media, based in part on the composition of cornsteep water, has already been mentioned, and the chemical changes occurring during fermentation of these media resemble those noted in the cornsteep-water

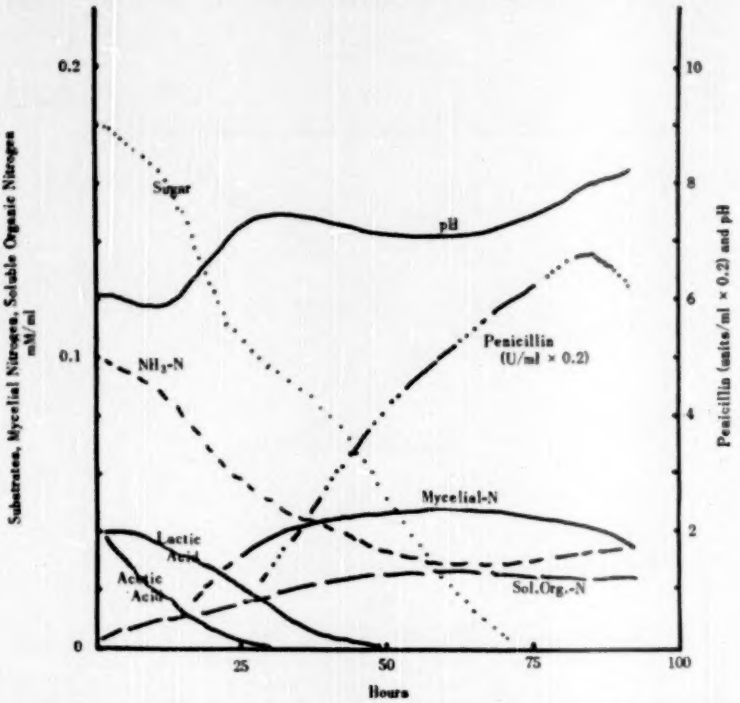


FIG. 6. Chemical changes occurring during fermentation of synthetic medium by *Penicillium chrysogenum* Wisconsin Q_{176} (Shaken Flask).
Medium composition (grams per liter): lactose, 22.5; glucose, 7.5; ammonium acetate, 3; ammonium lactate, 5; KH_2PO_4, 3; $MgSO_4 \cdot 7\ H_2O$, 0.25; $FeSO_4 \cdot 7\ H_2O$, 1; $CuSO_4 \cdot 5\ H_2O$, 0.005; $ZnSO_4 \cdot 7\ H_2O$, 0.02; Na_2SO_4, 0.5; $MnSO_4 \cdot H_2O$, 0.02; $CaCl_2 \cdot 2\ H_2O$, 0.05; distilled water to 1 liter. (Abstracted from Jarvis and Johnson (116)).

media, as indicated in Fig. 6. Use of synthetic media in these studies on the biosynthesis of penicillins seems advisable, as it probably permits easy reproduction of fermentations, an accomplishment that may be rather difficult when different natural media are used.

Most penicillin-producing cultures of *Penicillium chrysogenum* produce substantial quantities of proteolytic enzymes, amylases, phosphatases, amino acid oxidases, invertase and lipase during their growth on cornsteep-water media (84, 120, 128, 135, 139, 159, 179). While washed mycelium suspensions will produce some penicillin when incubated with a buffer–yeast extract solution (90), this approach to the mechanism of penicillin production has not received much attention. Apparently only preliminary studies have been made on the constitutive and extra-cellular enzymes of penicillin-producing molds, and no correlation between the activity of these enzymes and penicillin production has been postulated.

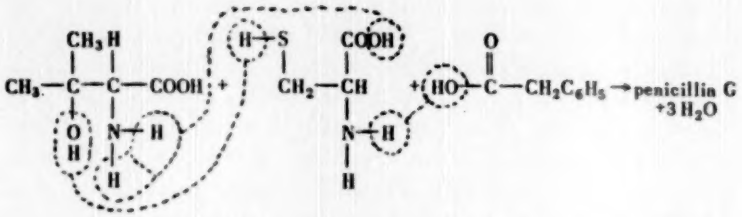
SEARCH FOR SUBSTANCES ACTING AS INTERMEDIATES IN BIOSYNTHESIS OF THE PENICILLIN. Mention has been made of the observations of Moyer et al. (46, 161) and Behrens et al. (9, 10, 11) that addition of phenylacetyl derivatives and other compounds resulted in increase in production of penicillins when added to growing cultures. These compounds have influenced the biosynthesis of penicillins from the R-group (see Table I), and, while successful and of considerable practical importance, the conversion of these compounds to penicillins has rarely exceeded 15% of that added and usually is of the order of 2% to 5%. It seems probable that the remainder of the preparations are oxidized or otherwise utilized by the mold as in animal tissues (24), and perhaps these studies may be interpreted to indicate that the fungus synthesizes excess quantities of the thiazolidine fragment.

Studies on the metabolism of sulfur compounds by penicillin-producing fungi have also been mentioned and have not indicated whether oxidized or reduced sulfur compound are more desirable for penicillin synthesis (5, 107, 108, 142). It is interesting to speculate that the variant of *Penicillium chrysogenum*, Wis. Q_{176}, which produces 3050 units of penicillin-G per ml., requires about .2 mg. per ml. of S to form enough thiazolidine portion of the molecule. This is nearly all the sulfur supplied by the 20 mg. of cornsteep water solids in the medium on a per ml. basis (144, 180).

Addition of penicilloic acid or other enzymatic (41, 161) or chemical breakdown products of penicillin to growing cultures has not resulted in increases in penicillin production, perhaps an indication that penicillinase action is not reversible by penicillin-producing organisms. While penicillinase activity has been noted

in mycelial preparations of *P. notatum* NRRL 1249.B21, the organism used in the surface culture process, no activity has been noted in cultures grown under submerged conditions, although penicillin potencies in the broth drop rather sharply after the pH of the fermentation passes 8.3 (91, 84, 135). The effectiveness of certain metallic ions in inhibiting the production of penicillin by certain cultures (*127*, *137a*) may be through activation of penicillinase as well as by the chemical catalysis of decomposition (*37*, 41).

Among the schemes suggested for the biosynthesis of penicillins is that of Hockenhull et al. (109) in which β-hydroxyvaline is thought to combine with cysteine and the penicillin precursor:



However, no evidence has been offered that these intermediates were synthesized by penicillin-producing cultures, although dimethylpyruvate was isolated from growing cultures and may have been an intermediate in the formation of the β-hydroxyvaline. The structural relationship of penicillin to glutathione has received some attention (68), but addition of glutathione to growing cultures has had no effect on penicillin production.

Biosynthesis of possible intermediates by cultures not producing the intact penicillin molecule has been studied without success. This has been approached by growing mixed cultures of nonproducers and producers (19), and by adding culture fluid from penicillin-producing cultures to these freshly inoculated fermentations (38, 39). A number of biochemical mutants of penicillin-producing cultures which require certain amino acids or growth factors for growth have been studied to determine whether these growth requirements influence the production of penicillin by these cultures (18, 19). These studies have not shown any correlation between a specific requirement and penicillin production.

SUMMARY

During the past ten years a multi-million dollar industry has developed, based on the mycological production of penicillin. Studies on factors influencing penicillin production have shown that selection of cultures, formulation of media, addition of intermediates in penicillin biosynthesis to the fermentations, and selection of optimum aeration and fermentation conditions play a part in the successful operation of this fermentation process. Although many studies have been made on the chemical changes occurring during the growth of the fungi and production of penicillin, little information has been gained on the mechanism of penicillin formation by fungi. However, these studies have helped in formulation of synthetic media which support growth and penicillin production equivalent to those obtained with media containing natural material.

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